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THE PREPARATION AND SOME PROPERTIES OF A LOW MOLECULAR WEIGHT RIBONUCLEIC ACID FROM BAKER'S YEAST

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SUMMARY

A low molecular weight RNA has been extracted from whole baker's yeast cells with phenol and compared with yeast S-RNA. Several properties which have been investigated are very similar for both products. Among these properties are the sedimentation constants, the chromatographic behavior on DEAE-cellulose columns, the formation of dinitrophenylhydrazones, the base composition, and the valine and adenylate accepting activity. The extraction procedure is adaptable to large scale preparations.

INTRODUCTION

The interest raised by a ribonucleic acid fraction of low molecular weight[1], able to accept activated amino acids and to transfer them to microsomes, made desirable a search for a readily available source, from which this material could easily be extracted. When living cells are homogenized and the homogenate is fractionated by differential centrifugation, this RNA is normally found in the 100,000 × *g* supernatant. Although it has been questioned whether the fraction of RNA able to accept amino acids (transfer RNA)[2] represents all of the supernatant or "soluble" RNA, even after prolonged centrifugation, transfer RNA from mammalian cells has usually been prepared from the 100,000 × *g* supernatant fraction. A similar technique has been used by TISSIÈRES *et al.*[3] for preparing transfer RNA from *E. coli*. While these techniques take advantage of the apparent intracellular localization of the material to be prepared, they unfortunately are long and not readily able to yield large quantities of RNA. PREISS *et al.*[4] have devised a different procedure, based on the low molecular weight of transfer RNA, to separate it from the bulk of *E. coli* RNA, extracted with hot detergent. In previous reports from this laboratory[5,6], it was shown that pressed baker's yeast can yield a fraction of RNA active as an acceptor of both activated amino acids and nucleoside triphosphates (ATP and CTP). The

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Abbreviations: RNA, ribonucleic acid; S-RNA, soluble ribonucleic acid; ATP, adenosine triphosphate; CTP, cytidine triphosphate; AMP, adenosine-5′-phosphate; DNPH, 2,4-dinitrophenylhydrazone; DEAE-cellulose, diethylaminoethyl-cellulose.

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present communication is concerned with a brief description of a technique, essentially derived from KIRBY's procedure[7], yielding (from pressed baker's yeast) an RNA fraction of low molecular weight. Properties of this RNA are also compared with those of the soluble RNA obtained after high speed centrifugation of disrupted yeast cells and with the properties of yeast ribosomal RNA. The conclusion is reached that the RNA extracted by this simple procedure is very similar to the S-RNA prepared by the more elaborate centrifugation-precipitation method previously employed.

METHODS

Preparation of the low molecular weight RNA

To 100 g of fresh pressed baker's yeast are added 200 ml of water and 300 ml of phenol–water (90:10, w/w). After 2 h of stirring at room temperature, the mixture is centrifuged and the aqueous layer recovered. The RNA is precipitated with cold ethanol–acetate, according to the method of KIRBY[7]. The precipitate is then dissolved in approx. 40 ml of water at room temperature. If any insoluble precipitate appears on standing at 0° for 10 min, it is removed by centrifugation for 10 min at 25,000 × *g* at 0°. From this point on, all operations are performed at or near 0°. The supernatant is stirred with 1 g of acid washed charcoal (Norite) for 20 min. The charcoal is eliminated by high speed centrifugation and filtration (if necessary) through a rapid filter paper (Whatman No. 41). The charcoal is washed with 5 ml of water. The RNA from combined charcoal wash and supernatant is precipitated with cold ethanol as before. The precipitate is dissolved in approx. 17 ml of water and any insoluble residue removed by centrifugation. The clear solution is extracted twice with 2-methoxyethanol according to the method of KIRBY[7]. The RNA is recovered from the upper layer by adding 0.1 volume of 20% potassium acetate, pH 5.0, and 2.5 volumes of cold ethanol. The precipitate, recovered by centrifugation, is dissolved in the minimum amount of water and dialyzed in the cold against three changes of distilled water (2 l each) over a period of 24 h. The yield is 70–80 mg of RNA.

Preparation of S-RNA and ribosomal RNA

The supernatant fraction of the yeast cells is prepared by rupturing the cells followed by centrifugation, according to CHAO AND SCHACHMAN[8]. At the end of the final 3-h centrifugation at 78,000 × *g*, the upper part of the supernatant is recovered. A loosely packed sediment, appearing on top of the tightly packed ribosome pellet, is left behind. S-RNA is isolated from the fraction by phenol extraction. A charcoal treatment is performed as previously described. For preparing ribosomal RNA, the surface of the ribosome pellet is washed free of poorly sedimented material, using CHAO AND SCHACHMAN's medium[8]. The ribosomes are then suspended in the same medium and treated with 90% phenol, and the RNA recovered, as described previously.

Enzyme preparations

In the case of the Ehrlich ascites enzymes, the S_4 fraction, described by HECHT *et al.*[9], is used.

For the preparation of yeast enzymes, pressed baker's yeast is homogenized with three times its weight of glass beads and 1 volume of CHAO AND SCHACHMAN's medium[8] using a Virtis 45 homogenizer, cooled with ice, at maximum speed for a total of 15 min. Two drops of octyl alcohol and 50 mg (for 30 g of yeast) of reduced glutathione are added at the start, and the homogenization is carried out in three periods separated by 3-min intervals. Under these conditions the temperature inside the homogenizer does not rise over 14°. From this point on, all operations are performed at or near 0°. One volume of medium is used to transfer the preparation to centrifuge tubes, and the supernatant is recovered after a 5-min centrifugation at 1500 × *g*. The glass beads are washed 3 times with 1 volume of medium, by centrifugation under the same conditions. The combined supernatant fractions are centrifuged at 78,000 × *g* for 3 h. The supernatant, removed from a loosely packed sediment is recovered and processed according to the following procedure:

Immediately after preparation, this supernatant (100 ml), the pH of which is approx. 6.5, is brought to pH 5.0 with 10 % acetic acid. About 5 min after the beginning of the operation, the precipitate which forms is collected by centrifuging at 25,000 × *g* for 5 min, and is washed once with 15 ml of 0.1 *M* sodium acetate, pH 5.0. The pellet is then suspended in 0.05 *M* Tris–HCl buffer, pH 7.6, containing 0.005 *M* $MgCl_2$, and the pH is adjusted to 7.6 with 1 *N* KOH. An insoluble residue is eliminated by centrifugation for 10 min at 25,000 × *g*. 1 g of streptomycin sulfate is added with stirring to the opalescent supernatant and the mixture is allowed to stand for 15 min with occasional stirring. The precipitate is eliminated by centrifugation for 10 min at 25,000 × *g*. The resulting supernatant is stirred with 4 g of a mixed bed resin preparation (AG 501-X8)* for 2 min. The resin and a protein precipitate are removed by centrifugation at 1,500 × *g* for 2 min, and are washed twice with 5 ml of water. The pH of the combined supernatants is brought to 7.6 by the addition of 0.05 volume of 1 *M* Tris–HCl buffer, pH 7.6, and the fraction is dialyzed overnight against 0.05 *M* Tris–HCl buffer, pH 7.6 and 0.005 *M* $MgCl_2$. A precipitate forms which is eliminated by centrifugation. The resulting enzyme preparation can be stored at —10°.

Enzyme activity determinations

Incorporation of [^{14}C]valine and [^{14}C]ATP into RNA was carried out according to previously published techniques[5,9]. Incubation mixtures for measuring [^{14}C]valine incorporation contained in a total of 1 ml: 10 μmoles of Mg-ATP, 0.5 μmole CTP, 5 μmoles of phosphopyruvate, 0.01 mg of phosphopyruvate kinase, 0.1 μmole of [^{14}C]valine ($4.5 \cdot 10^6$ counts/min/μmole), 100–200 μg RNA, 50 μmoles Tris–HCl buffer (pH 7.6), and 5 μmoles $MgCl_2$. For measuring [^{14}C]ATP incorporation 1 μmole of [^{14}C]ATP ($1.7 \cdot 10^5$ counts/min/μmole) was used, and the [^{14}C]valine was omitted. Other constituents were the same as used for the valine incorporation experiments. Incubation was carried out at 37°.

Nucleic acid procedures

RNA was determined spectrophotometrically at 260 mμ after 1 h hydrolysis in 1 *N* NaOH at room temperature, as described[9].

The ratios between adenylic acid, guanylic acid, cytidylic acid, uridylic acid and uracil-5-ribosyl phosphate were determined after alkaline hydrolysis according to a modification of the procedure of DAVIS *et al.*[10]. Minor base constituents were isolated and determined according to the method of DUNN[11].

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Amino acids attached to transfer RNA are eliminated by incubation in 0.1 *M* potassium carbonate buffer, pH 10.0, for 50 min at 37°, followed by extensive dialysis of the neutralized solution in the cold.

Reaction of RNA with periodate and 2,4-dinitrophenylhydrazine

The carbon atoms carrying vicinal 2′- and 3′-hydroxyl groups of the terminal ribose residues of transfer RNA, free of attached amino acids, are oxidized with 0.1 *M* sodium periodate in 0.05 *M* sodium acetate buffer, pH 5.0, for 1 h at room temperature in the dark. Most of the excess periodate is precipitated by the addition of 0.1 volume of 2 *M* KCl at 0°. After removal of KIO_4 by centrifugation, the supernatant is dialyzed. The aldehyde groups formed can be reacted with 2,4-dinitrophenylhydrazine under the following conditions: to 4 volumes of oxidized RNA solution, containing up to 5 mg of RNA/ml, are added 1 volume of 0.5 *M* sodium acetate buffer, pH 4.0, 3 volumes of 2-methoxyethanol free of peroxides and 4 volumes of a 1.2 % solution of 2,4-dinitrophenylhydrazine in 2-methoxyethanol. The mixture is incubated at 37° in the dark for 1 h. The excess reagent is eliminated by extraction 5 times with ethyl acetate, after first diluting the incubation mixture with 6 volumes of water. The 2,4-dinitrophenylhydrazone derivative of RNA (DNPH-RNA) is recovered by alcoholic precipitation. The extent of the reaction can be followed by measuring the extinction at 360 mμ, assuming a molecular extinction coefficient of 21,000 for the DNPH radical[12].

RESULTS

Sedimentation constants

The values of the sedimentation constants, determined in 0.01 *M* phosphate buffer, pH 7.0, at 59,780 rev./min in the analytical rotor of the Spinco model E ultracentrifuge, at a concentration of 0.005 % of RNA, are 4.3, 4.4 and 6.1 S respectively, for the RNA extracted by the phenol procedure from whole cells, the S-RNA, and the ribosomal RNA. The sedimentation patterns of the first two RNA samples do not give any indication of heterogeneity.

Solubility in concentrated salt solutions

While the RNA extracted from the ribosomes is completely insoluble in 1 *M* NaCl and in 65 % saturated ammonium sulfate[2], both the S-RNA and the RNA extracted from whole cells by the phenol procedure are completely soluble in 1 *M* NaCl. In 65 % saturated ammonium sulfate, the latter RNA is only partially soluble, but there is no difference in valine-accepting activity between the products recovered from the precipitate and from the supernatant.

Chromatographic behavior

The elution patterns obtained for the three samples of RNA by gradient elution chromatography on DEAE-cellulose columns are presented in Fig. 1. The RNA extracted from whole cells and the S-RNA both are eluted between 0.40 *M* NaCl and 0.56 *M* NaCl. The recovery is 94–97 % of the RNA originally put on the column. In the case of the ribosomal RNA, only approx. 7 % of the initial amount is eluted in this range of concentrations. The rest can be eluted with 0.1 *N* NaOH.

Nucleotide composition

The amounts of adenylic acid, guanylic acid, cytidylic acid, uridylic acid, uracil-5-ribose phosphate, thymine ribotide and 6-methylaminopurine ribotide, expressed relative to adenylic acid are given in Table I. The composition of the RNA extracted from whole cells is essentially identical to that of the S-RNA, within the limits of the precision of the method used. This composition is, however, very different from that of the ribosomal RNA.

Reaction with periodate and 2,4-dinitrophenylhydrazine

Evidence has been presented for the linkage of the amino acids to the 2'- or 3'-hydroxyl group of the ribose of the terminal adenine nucleotide of S-RNA[4, 5, 13]. When the amino acids have been removed from S-RNA by treatment with alkali, the terminal ribose of S-RNA contains hydroxyl groups on the 2'- and 3'-position of the ribose. These are available for oxidation by periodate to form a dialdehyde on the terminal ribose of the RNA chain.

It had previously been observed that the periodate reaction with S-RNA was blocked by the presence of amino acid in 3'- or 2'-ester linkage on the terminal ribose[4, 5, 13], and conversely that the attachment of amino acid to the terminal ribose of S-RNA was prevented by previous periodate oxidation of the S-RNA[4]. These two pieces of evidence point to the adjacent 2'- and 3'-hydroxyl groups of the terminal ribose unit of the S-RNA as being the site of periodate oxidation.

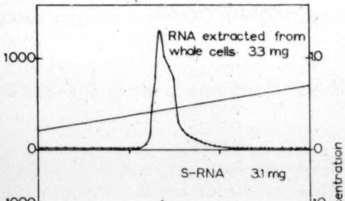

Fig. 1. Chromatography of RNA samples on DEAE-cellulose columns. Columns: 1.2 × 12 cm. A linear gradient of NaCl concentration is established using a mixer with 500 ml of 0.02 *M* potassium phosphate buffer, pH 7.7, containing 0.2 *M* NaCl and a reservoir with 500 ml of 0.2 *M* potassium phosphate buffer, pH 5.5, containing 1.2 *M* NaCl.

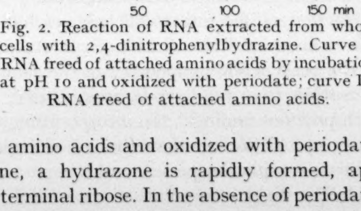

Fig. 2. Reaction of RNA extracted from whole cells with 2,4-dinitrophenylhydrazine. Curve I, RNA freed of attached amino acids by incubation at pH 10 and oxidized with periodate; curve II, RNA freed of attached amino acids.

When transfer RNA free of attached amino acids and oxidized with periodate is treated with 2,4-dinitrophenylhydrazine, a hydrazone is rapidly formed, apparently with these aldehyde groups of the terminal ribose. In the absence of periodate oxidation, there is essentially no reaction of S-RNA with dinitrophenylhydrazine during an incubation period of 2 h. The time course of the reaction is shown in Fig. 2.

It is essential that the nucleic acid samples be treated with 2-methoxyethanol

TABLE I

NUCLEOTIDE COMPOSITION OF RNA SAMPLES

	Molar ratio; adenylic acid = 10.0					
	Guanylic acid	*Cytidylic acid*	*Uridylic acid*	*Uracil-5-ribosyl phosphate*	*Thymine ribotide*	*6-methylamino-purineribotide*
RNA extracted from whole cells	16.1	15.9	10.2	2.2	0.32	0.19
S-RNA	15.8	15.3	10.8	2.1	0.29	0.17
Ribosomal RNA	10.7	7.7	10.1	0.3	< 0.04	< 0.04

prior to oxidation by periodate, since contamination by polysaccharide yields spuriously high values. Under these conditions Table II indicates that the attachment of DNPH to RNA was of the same order of magnitude in samples of RNA obtained by phenol extraction of whole cells and those of S-RNA obtained after differential centrifugation while the attachment of DNPH to ribosomal RNA was only 0.2 as much. These figures are not changed if the RNA samples are incubated with prostatic phosphatase before periodate oxidation. It is therefore unlikely that a phosphate monoester is present at the 2′- or 3′-position on this terminal ribosyl unit of the RNA.

TABLE II

REACTION OF PERIODATE OXIDIZED RNA WITH 2,4-DINITROPHENYLHYDRAZINE

	μmoles DNPH/mg RNA
Soluble RNA A	0.0663
Soluble RNA B	0.0614
RNA-phenol extract of whole cell III	0.0543
RNA-phenol extract of whole cell IV	0.0575
RNA-phenol extract of whole cell V	0.0875
Ribosome RNA B	0.0091

Labeling of RNA using $[^{14}C]$valine and $[^{14}C]$ATP

It had previously been shown by this laboratory[5,9] that S-RNA from liver or ascites cells was able to incorporate $[^{14}C]$AMP into the terminal position of the RNA chain, using $[^{14}C]$ATP as precursor and the appropriate enzymes. Similar samples of RNA are able to bind amino acid to the terminal adenine moiety of the RNA molecule. Table II indicates that using crude yeast enzyme, yeast RNA prepared by phenol extraction of the intact cell behaves like soluble RNA from yeast, in contrast to yeast ribosomal RNA which incorporates these compounds very poorly. These values in the yeast RNA samples are of the same order of magnitude as the incorporation in S-RNA from rat liver or ascites cells[5]. It is also noteworthy that, in agreement with previous findings[5], the incorporation of the mononucleotide precursor into RNA is 10–20-fold greater than that of a single amino acid precursor. This situation is consistent with the presence of a separate transfer RNA molecule for each amino acid. The slight incorporation observed in the ribosomal RNA may be explained as a slight contamination with S-RNA. This is in agreement with the chromatographic pattern observed in Fig. 1.

Table III also indicates that S-RNA and the RNA prepared by phenol extraction

TABLE III

COMPARISON OF LABELING OF VARIOUS TYPES OF YEAST RNA BY YEAST ENZYMES AND ASCITES ENZYMES

	mμmoles incorporated/mg RNA											
	[^{14}C]valine						*[^{14}C]ATP*					
	Yeast enzyme		*Ascites enzyme*		*Both*		*Yeast enzyme*		*Ascites enzyme*		*Both*	
Time (min)	*30*	*60*	*30*	*60*	*30*	*60*	*30*	*60*	*30*	*60*	*30*	*60*
Soluble RNA I	1.01	1.26	0.11	0.0	0.67	0.08	—	11.55	—	0.74	7.00	0.0
Soluble RNA II	0.80	0.80	0.07	0.0	0.37	—	—	8.48	—	0.34	3.25	0.0
RNA-phenol extract of whole cell I	1.07	1.29	0.94	1.12	1.30	1.18	—	21.10	—	20.70	19.15	22.05
RNA-phenol extract of whole cell II	1.43	1.69	1.48	1.45	1.36	1.47	—	25.70	—	25.15	26.80	27.80
Ribosome RNA	0.07	0.09	0.004	0.001	0.05	0.01	—	1.76	—	0.18	0.99	0.0

from the whole cells do not behave in the same way when the ascites enzyme is used. This enzyme is able to promote an incorporation of both [^{14}C]AMP and [^{14}C]valine into the RNA prepared from whole cells only. No incorporation occurs when S-RNA is used as an acceptor. This finding suggests that the S-RNA isolated after centrifugation of the yeast homogenate has lost some of its integrity, possibly due to an enzymic degradation during the lengthy isolation procedure. Apparently the crude yeast enzyme is able to restore this, while the ascites enzyme is not. The parallelism between [^{14}C]AMP and [^{14}C]valine incorporation also suggests that in agreement with previous findings using ascites S-RNA[5], when the RNA sample is unable to receive the terminal adenine, it is also unable to bind amino acid.

DISCUSSION

The properties of the RNA extracted from whole yeast cells and those of yeast S-RNA have been found to be similar in a number of respects. It is likely that, under the extraction conditions used, the cell wall is permeable only to small molecules and does not allow the extraction of ribosomes or ribosomal RNA. The yield of RNA obtained by our procedure (0.07–0.08 %) is compatible with the amount of S-RNA present in the original yeast cake (0.3 %)[8]. It has been shown that the [^{14}C]valine attached to the RNA extracted from whole yeast cells can be transferred to rat liver microsomes under suitable conditions[6]. Whether our RNA preparations are made only of transfer RNA remains to be determined by further work. We cannot at the present time exclude the possibility that our preparations contain a mixture of transfer RNA and of RNA molecules of similar molecular weight but performing a different function or originating from a partial degradation of ribosomal RNA.

We have already mentioned the similarity in activity between S-RNA from yeast and S-RNA from rat liver. If we compare our data on the base composition with those given by MAGASANIK[14] for rat liver S-RNA, a close similarity is again apparent. Comparable compositions have also been found for the S-RNA from *E. coli*[3], from Ehrlich ascites tumor cells and from guinea pig liver (*cf.* ref. 15). OTAKA *et al.*[16,17] have already described a preparation of yeast S-RNA. While their data on the nucleotide composition are roughly similar to ours, differences do exist on the guanylic acid and cytidylic acid contents. These differences could be due to the differences in the strains of yeast used. Our data do not permit an accurate determination of the molecular weight, but they would be in agreement with a value in the range of 20,000–30,000, which is comparable with the value already found by others for various S-RNA preparations[3,9,11], but definitely smaller than the value of 50,000 reported by PREISS *et al.*[4] for *E. coli* S-RNA.

The fact that the incorporation of [^{14}C]valine into the samples of S-RNA prepared by us is dependent on the incorporation of AMP, indicates that the molecules are not only free of amino acids but even free of terminal AMP residues and therefore are non-functional as isolated. We cannot state at the present time whether this is a consequence of a partial degradation occurring in the course of the preparation or whether this is due to the low levels of the nucleotide and amino acid pools in the resting cells we are using for our preparations.

The chief virtue of the present method is that an RNA, capable of accepting and transferring amino acids in an *in vitro* amino acid incorporation system, may be readily prepared from commercial baker's yeast in large quantities.

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DEOXYPENTOSE NUCLEIC ACIDS

XIII. THE DENATURATION OF DEOXYRIBONUCLEIC ACID IN AQUEOUS SOLUTION: A TEST FOR THE IRREVERSIBILITY OF THE CRITICAL CONCENTRATION PHENOMENON

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SUMMARY

Evidence has been reported which indicates that dilution of a salt free DNA solution below a critical concentration zone ($4\text{-}50 \cdot 10^{-5}$ *M*) results in denaturation. This process is irreversible with respect to the specific hydrogen bonds present in the native structure, but is thought to be partially reversible with respect to non-specific hydrogen bonds. The non-specific hydrogen bonds would form above the critical concentration zone between polynucleotide strands at points of close approach. This latter state can be reached by reconcentration to above the critical concentration zone and also by addition of salt to solutions below the critical zone.

The structural change resulting from dilution below the critical concentration zone appears similar to that observed for saline DNA solutions which undergo denaturation when heated through a critical temperature range. In both instances the broad ranges of denaturation constraint can be interpreted in terms of hydrogen bond strength heterogeneity.

INTRODUCTION

Previous measurements of the electrical transport number and conductivity of aqueous solutions of DNA[1,2] have been interpreted in terms of a denaturation process. The critical concentration zone that is observed in the variation of the specific conductivity with DNA concentration is now considered to arise from denaturation by dilution. Above this zone (greater than $50 \cdot 10^{-5}$ *M* DNA) essentially native DNA exists, while at concentrations lower than $4 \cdot 10^{-5}$ *M* the denatured structure predominates. An alternative, less likely, explanation of the critical concentration phenomenon involves aggregation of DNA molecules above the critical zone[1]. It is improbable that specific binding would be involved in such an aggregating system and therefore it would be expected that such a system would be completely reversible on dilution and reconcentration through the critical concentration. The more likely

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Abbreviation: DNA, deoxyribonucleic acid.

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process of denaturation would be irreversible in view of the specific nature of the hydrogen bonds, which break during denaturation and are not reformed on restoring the original conditions.

Although the explanation involving denaturation must be favoured in view of the tendency for denaturation at low ionic strength[3–5], the ultimate decision on this question rests on testing the reversibility or irreversibility of the critical concentration phenomenon. An endeavour has been made in the present investigation to apply such a test. Although complicating factors are present, it appears that the process is essentially irreversible and can therefore be satisfactorily explained by a denaturation process.

EXPERIMENTAL

A description of the preparative histories of the calf thymus DNA samples used in this investigation has already been given[6]. The conductivity bridge was identical to that already described[1] and the technique used to determine the critical concentration has also been reported[2]. All DNA concentrations were determined by phosphorus analysis.

RESULTS AND DISCUSSION

It is now well established that the anomalous low absorptivity (measured at 2590 Å) of native DNA originates from some property of the closely stacked and hydrogen bonded nitrogen bases that occur on the inside of the DNA double helix. As denaturation involves the destruction of this orderly arrangement of bases, it should be accompanied by increased u.v. absorption. This has been experimentally observed in many investigations on the denaturation of DNA. However, it also appears that the increase in absorptivity can in some respects be a rather insensitive measure of the initial stages of denaturation[5].

An essentially native DNA solution (initially dissolved in water at high DNA concentration) has an atomic extinction coefficient referred to the phosphorus concentration, $\varepsilon(P)$, of 6660 (see ref. 6). This value was obtained by dilution of a concentrated aqueous solution to $5 \cdot 10^{-5}$ *M* DNA in the presence of salt to give a final sodium chloride concentration of 0.1 *M*. A salt free solution has a similar value at high concentrations[5] (greater than $9\text{–}10 \cdot 10^{-4}$ *M* DNA). At lower DNA concentrations a discontinuity has been observed[1,5,7,8] in the variation of $\varepsilon(P)$ with concentration, which results in an extinction coefficient of 8200 (see ref. 5) below the critical concentration zone. Values of 8500 and 8400–8000 have been found for similar solutions of preparations 4 and 7 in the present investigation. This increase in extinction coefficient has been associated with denaturation[1,2,5]; however, the observed increase is complicated by the fact that the addition of salt to such a solution lowers the absorptivity to an intermediate value. It would therefore appear that both irreversible and reversible changes comprise the structural transition responsible for the critical concentration. As the addition of salt to a solution below the critical concentration zone results in a partial lowering of the extinction coefficient, it will be assumed that the value so obtained represents the true irreversible increase brought about by dilution below the critical zone.

Previous studies[1,2] have shown that a critical concentration zone also exists in the variation of the specific conductivity with DNA concentration. It is of interest to compare the critical zone over which the irreversible increase in u.v. absorption occurs with that found from the conductivity measurements. Fig. 1 shows the irreversible increase in extinction coefficient that is observed at low DNA concentrations. Curve a (Fig. 1) represents the values obtained at the various DNA concentrations by dilution of stock solution ($3 \cdot 10^{-3} M$ DNA) in the presence of salt; curve b represents the large, partially reversible increase, that accompanies dilution in the absence of salt; and curve c was obtained by dilution of a stock solution with water only, followed by the addition of solid salt to yield 0.1 *M* NaCl, the solution being then diluted to $5 \cdot 10^{-5} M$ DNA with 0.1 *M* NaCl and the absorption determined. Curve c thus represents the irreversible increase that accompanies dilution of a salt free solution below the critical concentration zone. It can be seen that the irreversible increase begins at concentrations lower than $10^{-3} M$ DNA. This is in agreement with the partially reversible increase shown in curve b. Previous work by CAVALIERI, ROSOFF AND ROSENBERG[5] has shown that this partially reversible increase begins at concentrations below $9–10 \cdot 10^{-4} M$ DNA. As the critical concentration phenomenon is at least in part irreversible it appears that the process originates from denaturation.

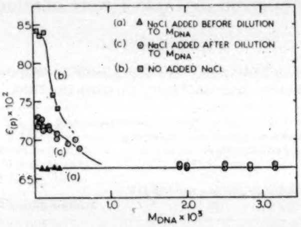

Fig. 1. Increase in atomic extinction coefficient accompanying dilution of DNA.

The concentration at which denaturation begins, as judged by the above results, is higher than that found from the conductivity measurements. It is concluded that dissolution of aqueous DNA solutions at concentrations below the critical concentration in the absence of salt results in a 27 % increase in extinction coefficient. The irreversible component, however, is only 11 %. These figures represent the average of several results. These values are to be compared with those of 25 % and 11 % respectively reported by THOMAS[3] for solutions at low DNA concentration and ionic strength. Thermal denaturation results in an irreversible increase of 15 % (see ref. 9). Differences in extinction coefficient of denatured DNA (usually measured at room temperature) could possibly arise because of the temperature dependence of the u.v. absorption of the denatured species[10]. Similar measurements were made on two further DNA preparations (preparations 1 and 5) which contained salt impurity; the irreversible increases were only 6 % and 3 % respectively. The smaller increases are understandable when the great sensitivity of the critical concentration to salt is considered[2].

The above experiments confirm the previous conclusions that irreversible increases in extinction coefficient occur in salt free solutions at low DNA concentrations. The change appears to originate from a structural transition from essentially native to denatured DNA as the polyion concentration is decreased through the critical concentration zone. It is thought that this transition is similar to that observed on heating saline solutions of DNA; in this case a critical temperature range exists within which denaturation takes place[9].

In order to test the irreversibility of the critical concentration phenomenon observed in the conductivity measurements, it is necessary to prepare a solution at high DNA concentration ($3 \cdot 10^{-3}$ *M*), which had previously been subjected to dissolution below the critical zone. This was done by freeze drying aqueous solutions ($5 \cdot 10^{-5}$ *M* DNA) and redissolving in water to give $3 \cdot 10^{-3}$ *M* DNA. This solution could then be studied for irreversible changes by conductivity measurements as compared with solutions at a similar concentration not first diluted below the critical zone.

Before any confidence can be placed in such a comparison, further factors must be considered. It is necessary to determine just how relevant the observed irreversible increase in extinction coefficient is to the problem. If, for instance, the sample that had been diluted and reconcentrated from below the critical concentration is again diluted to $5 \cdot 10^{-5}$ *M* DNA in the absence of salt, would a further irreversible increase in extinction coefficient occur? The results shown in Table I suggest that no further changes accompany a second dilution below the critical concentration zone. The atomic extinction coefficients given in Table I were obtained in the following way:

TABLE I

IRREVERSIBLE INCREASE IN ATOMIC EXTINCTION COEFFICIENT ACCOMPANYING DILUTION BELOW THE CRITICAL CONCENTRATION

Preparation	*ε(P) of DNA solutions (measured in 0.1 M NaCl)*			
	*Initially native solution at $3 \cdot 10^{-3}$ M DNA (1)** *Solution diluted to $5 \cdot 10^{-5}$ M DNA (2)*		*Denatured by one dilution below critical concentration and freeze dried to $3 \cdot 10^{-3}$ M DNA (4**)* *Solution diluted to $5 \cdot 10^{-5}$ M DNA*	
	Salt added first	*Salt added last*	*Salt added first*	*Salt added last*
4	6710	7300	7350	7350
7	6610	7350	7400	7380
5**	6500	6740	7010	7000

* Numbers refer to solutions shown in Table II.
** Preparation 5 contained salt impurity.

The ε(P) values given in the first two columns were obtained by the addition of salt *before* (column 1) and *after* (column 2) dilution of an aqueous stock solution to $5 \cdot 10^{-5}$ *M* DNA. Column 1 gives the ε(P) of the native sample, while the second column shows the irreversible increase that accompanies a single dilution below the critical concentration. The final two columns refer to similar measurements made on a solution denatured by dilution below the critical zone followed by freeze drying to give the stock solution at $3 \cdot 10^{-3}$ *M* DNA. Column 3 therefore again represents the irreversible increase associated with a single dilution below the critical concentration (any damaging effect due to the freeze drying procedure would show up here). Column 4, on the other hand, was obtained by dilution of the reconcentrated solution to $5 \cdot 10^{-5}$ *M* DNA *before* the addition of salt and therefore represents a DNA sample subjected twice to the influence of the process operating at the critical concentration. No further denaturation by dilution was found. In addition freeze drying had no measurable effect on the ε(P) of a concentrated aqueous solution of native DNA. Table II shows the various solutions used in this investigation. From the constancy of the irreversible increase in ε(P) it appears that the denaturation produced by dilution below the critical concentration represents a well defined state and not just one of a series of denatured states. The average ε(P) obtained for six different batches of DNA, irreversibly changed by dilution followed by freeze drying, was found to be 7340 ± 60 (measured in 0.1 *M* NaCl at room temperature). These figures are shown in Table III.

TABLE II

DNA SOLUTIONS REFERRED TO IN THE TEXT

Reference number	*Solution*
(1)	Solution of native DNA dissolved in water at $3\cdot10^{-3}$ *M* DNA
(2)	Solution (1) diluted with water to $5\cdot10^{-5}$ *M* DNA
(3)	Solution (2) freeze dried
(4)	DNA from (3) dissolved in water to give $3\cdot10^{-3}$ *M* DNA
(5)	Solution (4) adjusted to 1 *M* NaCl with solid salt
(6)	DNA precipitated from (5) with ethanol and dried
(7)	DNA from (6) redissolved in water to give $3\cdot10^{-3}$ *M* DNA

TABLE III

ATOMIC EXTINCTION COEFFICIENT OF DNA DENATURATED BY DILUTION

Preparation	*Number of batches prepared*	*ε(P) of DNA denatured by dilution followed by freeze drying (measured at room temperature in 0.1* M *NaCl)*
2	2	7420
4	1	7450
7	3	7340

A second complication that must be considered is whether any further irreversible effect is caused by freeze drying, which might not be detected by the absorption measurements given in Table I. Two observations have been made which indicate that something of this nature possibly occurs. Firstly, the viscosity of a denatured solution (diluted to $5\cdot10^{-5}$ *M* DNA in the absence of salt) is about 10 times as great as that of a similar solution obtained by freeze drying and dilution to $5\cdot10^{-5}$ *M*. However, this anomaly may not be due to the freeze drying process, because the viscosity of an aqueous solution diluted below the critical concentration appears to decrease with time[11], as compared with a similar solution reconcentrated and rediluted. Secondly, a sample that had been denatured by dilution and reconcentrated was adjusted to 1 *M* NaCl and precipitated with ethanol. This sample when dissolved in 0.2 *M* NaCl gave further irreversible changes over and above that shown by a solution denatured by dilution only. The relationship between these various solutions is shown in Table II. The changes in both ε(P) and S_{20} are shown in Table IV. Unfortunately it is not possible to precipitate DNA with ethanol at DNA concentrations of $5\cdot10^{-5}$ *M* and therefore it is not possible to determine whether these further irreversible changes, which become apparent on precipitation, arise from the freeze drying or the denaturation processes. It was also observed in the sedimentation study just mentioned that u.v. absorbing, non-sedimenting species were present in the precipitated and redissolved material, thus indicating that precipitation of denatured and freeze dried DNA is accompanied by degradation.

TABLE IV

IRREVERSIBLE CHANGES FOLLOWING PRECIPITATION OF DNA PREVIOUSLY DENATURATED BY DILUTION

Preparation	*Solutions denatured by dilution and then reconcentrated (4)**		*Solutions denatured by dilution, reconcentrated, precipitated and redissolved (7)**	
	S_{20}**	$\varepsilon(P)$***	S_{20}**	$\varepsilon(P)$***
2	19	7450	9–10	9900
4	16	7330	11–14	9900
7	17	7340	7–9	8100

* Numbers refer to solutions shown in Table II.
** Sedimentation of $2 \cdot 10^{-4}$ *M* DNA in 0.2 *M* NaCl.
*** Absorption measured in 0.1 *M* NaCl at room temperature and 2590 Å.

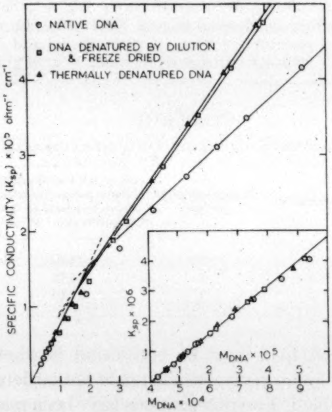

Fig. 2. Comparison of the critical concentration phenomena displayed by native DNA, DNA denatured by dilution and thermally denatured DNA solutions. Inset plot shows the conductivity at the lower concentrations.

A comparison of the critical concentration phenomenon, as judged by conductivity measurements, between initially native DNA and DNA diluted below the critical concentration followed by freeze drying, will now be given. A description of the two solutions used in this comparison (1 and 4) is given in Table II. Fig. 2 shows the variation of conductivity with concentration of the two solutions in addition to that of heat denatured DNA reported earlier[2]. At low concentrations, below the critical concentration zone, the three samples have similar equivalent conductivities, which suggests that a similar kinetic unit is involved in each case. This lends strong support to the suggestion that the critical concentration originates from a structural transition from native to denatured DNA. Below the critical zone the three samples are all in the denatured state, viz., the initially native sample which has been denatured by dilution, the sample initially denatured by dilution and freeze dried, and the thermally denatured sample. At DNA concentrations above the critical zone both the thermally denatured solution and the material initially dissolved below the critical zone followed by freeze drying, have similar conductivities and conductivity concentration dependence, implying again that the kinetic unit of the freeze dried solution is in the denatured state. In both cases the conductivity is higher, at concentrations above the critical zone, than that shown by native DNA solutions.

If the critical concentration phenomenon arose entirely from the denaturation of DNA it would be expected that initially denatured solutions would not display any discontinuity at all in the conductivity measurements; however, as can be seen from Fig. 2, this is not so. Although the magnitude of the effect is reduced, the discontinuity is still apparent. Two explanations appear possible, denaturation may not be complete; or denaturation as followed by conductivity measurements is partly reversible. The former explanation does not seem likely, because the absorptivity measurements given in Table I show that a further dilution below the critical concentration does not yield further irreversible increases in $\varepsilon(P)$. Also, the discontinuity that exists in plots of O.D. (measured at 2590 Å) versus DNA concentration in the absence of added salt is not present in the denatured samples (Fig. 3).

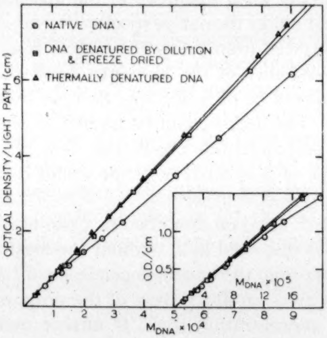

Fig. 3. U.V. absorption of native DNA, DNA denatured by dilution and thermally denatured DNA. Inset plot shows the O.D. at the lower concentrations.

The partial reversibility of this process, as observed in the conductivity measurements, can possibly be explained by the reformation of non-specific hydrogen bonds. These reformed bonds would have to be such that no resemblance to spiral nitrogen base stacking resulted (to be compatible with the absence of the discontinuity in the u.v. measurements). Intermolecular hydrogen bonding may also have to be considered.

A tentative explanation of the critical concentration phenomenon can now be put forward.

1. At high aqueous DNA concentrations (greater than $50 \cdot 10^{-5}\,M$ by conductivity measurements and greater than $100 \cdot 10^{-5}\,M$ by absorptivity measurements), an essentially native DNA molecule exists. Hydrogen bond cleavage, if it occurs, is reversible. It has been shown that under these conditions the kinetic unit possesses a charge fraction (charge per phosphorus atom) of 0.40. A large proportion of the gegenions are therefore held by the nucleate ion in aqueous solution. The careful work of CAVALIERI, ROSOFF AND ROSENBERG[5] has shown, however, that the viscosity,

as measured in 0.2 M NaCl, of DNA, initially dissolved in water at concentrations as high as $20 \cdot 10^{-4}$ M DNA, has already suffered a small but significant decrease as compared with a similar sample never dissolved in solvents of low ionic strength. Similarly it has been shown[6] that a 1.5 % increase in $\varepsilon(P)$s accompanies dissolution of native DNA in water at concentrations as high as $3 \cdot 10^{-3}$ M. It must therefore be concluded that a small amount of denaturation is inevitable when salt free DNA solutions at high concentration are studied.

2. Dilution below the critical concentration zone (lower than $4 \cdot 10^{-5}$ M DNA) in the absence of salt results in the denaturation of DNA (total hydrogen bond cleavage, or rupture of more than a critical number of bonds). The kinetic unit resembles the heat denatured material only if the latter is also diluted below the critical concentration zone in the absence of salt. Solutions of DNA that have been denatured by dilution have a high charge[1] and it would appear that charge fractions approaching unity are produced by this process. The differences in other electrical properties have already been described[1]. It is thought that the high charge on the denatured species may possibly counter the collapsing tendency that must accompany denaturation and allow the denatured molecule when dissolved in the absence of salt to take up an extended configuration, not unlike the native species.

3. Solutions reconcentrated from below the critical concentration zone are characterized by partial reversibility of the hydrogen bond cleavage process. The reformed hydrogen bonds would be of a non-specific type bearing no resemblance to "native hydrogen bonds". The bonds could be formed at points of closest approach between portions of the polynucleotide chain that were relatively far apart in the native structure. This type of denatured molecule would correspond to the samples thermally denatured in the presence of salt[9].

It should be noted that situation described in 3 can also be attained by the addition of salt to the species discussed in 2, without the need for reconcentrating the DNA solutions. This follows from the known dependence of the critical concentration on ionic strength[2]. Fig. 4 gives an illustration of the proposed structural transition occurring at the critical concentration zone. If further investigations confirm the above findings, then in the authors' opinion situation (2) should be a most fruitful starting point for a further process designed to dissociate the twin polynucleotide strands.

An examination of Fig. 2 shows that denaturation by dilution occurs over a

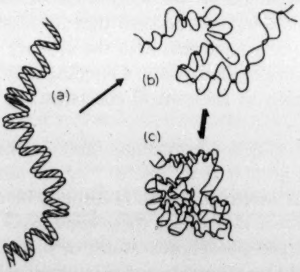

Fig. 4. Illustration of the proposed structural transition accompanying dilution below the critical concentration zone (a) ⟶ (b), and reconcentration to higher concentrations (b) ⟶ (c).

broad concentration range ($4\text{-}50 \cdot 10^{-5}\,M$), which would not be entirely compatible with the cooperative melting out of all the native hydrogen bonds. Moreover, it can be seen from Fig. 1 that the amount of denaturation (measured as an increase in $\varepsilon(P)$) is dependent on the concentration. Dilution to concentrations within the critical concentration zone produces various amounts of denaturation, which are always less than that produced by dilution to below $4\text{-}5 \cdot 10^{-5}\,M$ DNA. This is taken to mean that the hydrogen bond strengths of the native structure are heterogeneous, the weaker bonds permitting some denaturation at the higher concentrations within the critical zone, while the stronger bonds do not rupture until lower DNA concentrations. This state of affairs is quite analogous to the critical temperature zone within which native saline DNA solutions undergo denaturation[9], the existence of a broad temperature zone having been, in this instance, interpreted in terms of hydrogen bond heterogeneity. As with denaturation by dilution, the amount of thermal denaturation depends on the applied constraint, the weaker hydrogen bonds melting out at the lower temperatures within the critical temperature zone[9]. These observations, coupled with the demonstrated double boundaries in sedimentation experiments on DNA solutions within the critical concentration zone at low ionic strength[12], would indicate that the hydrogen bond strength heterogeneity is on an intermolecular scale.

No explanation can yet be given for the energetic reason for denaturation by the apparently simple process of dilution in the absence of salt. One line of approach will be to explore the possibility that the process involving hydrogen bond cleavage (and also non-specific hydrogen bond formation) is influenced by the condition of the "ice-like" structure of the water surrounding the DNA molecules. It can be imagined, for instance, that denaturation by dilution could result from the competitive effect of water–base hydrogen bonding on the native base–base hydrogen bonds. Native DNA is thought to have an ordering effect on the surrounding water[13,14] and, if this effect was large enough under the condition of high dilution of DNA, then the above possibility would be feasible.

ACKNOWLEDGEMENTS

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POTATO PHOSPHORYLASE

I. PURIFICATION, PHYSICOCHEMICAL PROPERTIES AND CATALYTIC ACTIVITY

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SUMMARY

A large scale preparation for the isolation of potato phosphorylase is described. Electrophoresis, sedimentation and diffusion measurements have been employed for the investigation of the homogeneity of the enzyme. The sedimentation coefficient was $7.9_4 \cdot 10^{-13}$ sec, the diffusion coefficient $3.7_6 \cdot 10^{-7}$ cm^2 sec^{-1} and the calculated molecular weight, 207,000.

The turnover number at 30° at pH 6.3 was 9400 moles of glucose-1-phosphate per mole of protein/min. MICHAELIS constants, pH optimum, the effect of metals and the stability of the enzyme under various conditions have been determined. The enzyme preparation did not contain any nucleotide and was not activated by 5'-adenylic acid or other nucleotides.

INTRODUCTION

In order to determine the molecular weight and the phosphate content of potato phosphorylase, it was necessary to prepare a sufficient quantity of enzyme in homogeneous form. Existing methods for the purification of this enzyme[1,2] were not suitable for this purpose. A convenient procedure which yields about 80 mg of enzyme protein from 5 kg of potatoes is described in this paper.

The properties and kinetic behavior of potato phosphorylase have been studied by several investigators with partially purified enzyme preparations. It seemed desirable to repeat some of these observations with the highly purified enzyme preparation described in this paper.

EXPERIMENTAL

Glucose-1-phosphate (G-1-P) as the dipotassium salt, was obtained from Schwarz Laboratories. Potato amylose and amylopectin were kindly contributed by STEIN, Hall and Co., Inc., New York, N.Y.

Abbreviations used: G-1-P, glucose-1-phosphate; TCA, trichloroacetic acid; 5'-AMP, 5'-adenylic acid; 2'-AMP, 2'-adenylic acid; 5'-CMP, cytidine-5'-phosphate; 5'-GMP, guanosine-5'-phosphate; 5'-IMP, inosine-5'-phosphate; 5'-UMP, uridine-5'-phosphate.

Enzyme assays were carried out at pH 6.3 in a reaction mixture (1 ml) containing 0.1 M citrate buffer, 0.01 M G-1-P and 0.76 % amylopectin. After 5 min at 30°, the reaction was stopped by the addition of 0.5 ml of 5 % TCA and the pH adjusted to about 4 by the addition of 2 ml of 0.1 M sodium acetate solution. The amount of inorganic P formed by the enzymic reaction was determined by the method of LOWRY AND LOPEZ[3]. A suitable blank was prepared for each series of measurements by incubating the reactants without G-1-P and addition of the latter after the protein precipitation.

Under the conditions described, the reaction was first order with respect to G-1-P. Enzyme units were defined as $K \times 1000$, where K is the first order velocity constant ($\log_{10}$, $\min^{-1}$). These are the same units which have been used in this laboratory to express the activity of muscle phosphorylase[4]. K was proportional to enzyme activity over a 20-fold dilution of the enzyme. It should be noted that the concentration of G-1-P (0.01 M) in the assay of the potato enzyme was lower than in the case of the muscle enzyme (0.016 M). Specific activity was expressed as units/mg of protein.

Protein in crude enzyme preparations was determined by the biuret method of GORNALL *et al.*[5]. In more highly purified preparations, protein concentration was calculated from O.D. measurements at 280 mμ in the Beckman spectrophotometer. The log I_0/I reading for a solution containing 1 mg of highly purified enzyme protein/ml in a cell of 1 cm light path was 1.29, the protein concentration having been determined by drying to constant weight.

Purification procedure

Potato juice: Idaho potatoes, obtained in the open market, were kept in the cold room. The washed potatoes were sliced and homogenized in the Waring blendor for about 1 min in a solution of 0.5 % $Na_2S_2O_4$–0.5 % sodium citrate (100 ml/kg of potatoes). The homogenate was passed through 4 layers of gauze and the residue squeezed out in a laboratory press. In order to settle out the starch, the combined potato juice was allowed to stand for 1 h in the cold room (3°). All further steps were carried out in the cold room. pH adjustments during fractionation with solid ammonium sulfate were made by addition of ammonia.

Precipitation-adsorption by amylose: Inert protein was removed by the addition of 20 g of solid ammonium sulfate to 100 ml of the starch-free potato juice at pH 6.5. After standing for several hours or overnight, a clear filtrate was obtained by centrifugation and filtration with filter aid (Celite). To the filtrate was added slowly, with sufficient stirring so as to avoid the formation of lumps, 1 g of finely powdered amylose/100 ml of fluid. After stirring for 1 h, 18 g of solid ammonium sulfate/100 ml was slowly added at pH 6.5 and the stirring continued for another hour. The centrifuged precipitate was extracted with 5 successive portions of 50 ml each of 1.8 M ammonium sulfate solution, pH 6.5, per kg of potatoes used. The residue was then eluted with successive portions of 50 ml each of 0.1 M citrate buffer pH 6.5 per kg of potatoes used until most of the activity was extracted.

Second ammonium sulfate fractionation: To the combined eluate was slowly added 20 g of solid ammonium sulfate/100 ml at pH 6.5. After centrifugation, 18 g of solid ammonium sulfate/100 ml was added to the supernatant fluid. The precipitate was collected by centrifugation and was suspended in a small amount of 0.002 M potassium phosphate buffer, pH 6.8, to give a protein concentration of about 10 %. This suspension was dialyzed against large volumes of the same buffer (saturated with thymol) for about 2 days with 3 changes of the buffer solution. The precipitate which formed during the dialysis was centrifuged off and discarded. The protein concentration of the supernatant fluid was about 6.5 %.

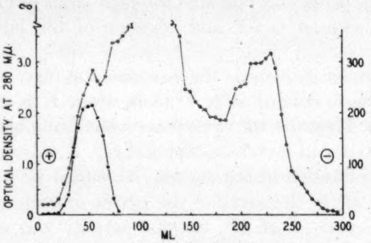

Fig. 1. Electropherogram of the first zone electrophoretic separation. ○—○, protein concentration (O.D. at 280 mμ); ●—●, phosphorylase activity expressed in Klett units. 700 mg of the 2nd ammonium sulfate fraction, 38 mA for 22 h.

First column electrophoresis: The apparatus used and procedure followed were based on those described by PORATH[6]. About 700 mg of protein solution in 0.002 M phosphate buffer, pH 6.8, was applied to a column 600 × 25 mm which contained 70 g of Munktell's cellulose powder for electrophoresis. The protein zone was moved down about 1 cm from the top by the addition of buffer solution. The top of the column was connected to the cathode, the bottom to the anode and a current of 38 mA was applied for 21 to 22 h. The column then was eluted with the same buffer solution at a flow rate of about 50 ml/h. Fractions of 5 ml each were collected for the measurement of protein concentration at 280 mμ and for the determination of enzyme activity. One of the experiments is shown in Fig. 1.

The combined active fractions were treated with solid ammonium sulfate; 28 g/100 ml was added and after 1 h the suspension was centrifuged and 12 g was added/100 ml of the supernatant fluid. The resulting precipitate was collected by centrifugation, suspended in a small amount of 0.002 M phosphate buffer pH 6.8 and dialyzed against the same buffer for 1 day with 2 changes of the buffer solution.

Second column electrophoresis: This was carried out in the same manner as the first zone electrophoretic separation; one experiment is shown in Fig. 2.

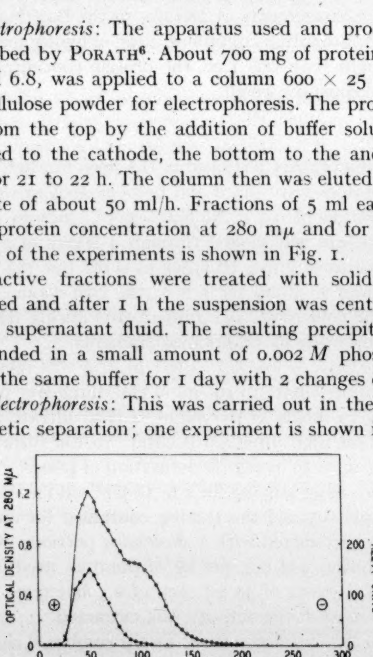

Fig. 2. Electropherogram of the second zone electrophoretic separation. ○—○, protein concentration (O.D. at 280 mμ); ●—●, phosphorylase activity expressed in Klett units. 65 mg of the first zone electrophoretic fraction, 38 mA for 22 h.

TABLE I

PURIFICATION OF POTATO PHOSPHORYLASE*

Fraction	*Total protein (mg)*	*Total units*	*Units per mg*	*Recovery (%)*
1. Potato juice	29,800	848,000	28.5	(100)
2. Precipitation-adsorption	4,160	705,000	170	74.5
3. 2nd ammonium sulfate fractionation	2,560	487,000	191	57.5
4. 1st column electrophoresis	280	193,000	687	22.3
5. 2nd column electrophoresis	80	125,000	1560	14.7

* From 5 kg of potatoes.

The combined active fractions were fractionated with ammonium sulfate as described in the preceding section and the final precipitate was dissolved in 0.1 *M* citrate buffer pH 7.

The procedure outlined results in about a 55-fold purification of the enzyme, starting with starch-free potato juice. An example is given in Table I. It should be emphasized that the yield and the specific activity of the final product depend to some extent on the activity of the starting material.

Potato phosphorylase could also be isolated by gradient elution with 1 *M* KCl–0.002 *M* Tris buffer, pH 7.5 from DEAE cellulose columns. The enzyme appeared in the last fractions and the degree of purification achieved was about the same as by zone electrophoresis.

Attempts to crystallize the purified enzyme preparations were not successful; in particular, the ammonium sulfate method described by BAUM AND GILBERT[2] failed to yield a crystalline product.

Physical measurements

The purified preparation obtained from the second zone electrophoresis was used in these experiments.

Electrophoresis: Measurements were made in the 2-ml open cells of the Perkin-Elmer apparatus, Model 38A. The protein solutions were dialyzed for 24 h with stirring against 3 l of potassium phosphate buffer of ionic strength 0.1 at pH values from 6.6 to 8.0 at 1°. Three of the electrophoretic patterns are shown in Fig. 3. It

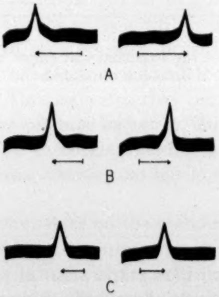

Fig. 3. Electrophoretic patterns of potato phosphorylase. A, pH 6.9; B, pH 7.2; C, pH 7.5; the bases of the arrows indicate the initial boundary.

except Hg^{++}. Examples of the effect of heavy metals on enzyme activity in glycerophosphate buffer are shown in Fig. 7.

TABLE II

STABILITY OF POTATO PHOSPHORYLASE

Incubated in 0.05 *M* citrate buffer, except where noted.

Period of incubation	*pH*	*Temperature*	*Percent loss of enzyme activity*
5 min	6.3	50	10
5 min	6.3	55	30
5 min	6.3	60	90
5 min	6.3	65	100
17 h	5.9–7.7*	30	0
17 h	7.7–8.7*	30	0
10 min	5.3	30	10
60 min	5.3	30	20
10 min	4.9	30	75
60 min	4.9	30	100
10 min	9.3**	30	20
60 min	9.3**	30	35

* 0.05 *M* Tris buffer.
** 0.05 *M* glycine buffer.

Metal binding agents (CN^-, histidine, 8-hydroxyquinoline, Versene, $1 \cdot 10^{-3}$ *M*) had no effect on enzyme activity. No evidence could be obtained that potato phosphorylase requires a metal for its activity.

ACKNOWLEDGEMENTS

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POTATO PHOSPHORYLASE

II. PHOSPHATE AND SULFHYDRYL GROUPS

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SUMMARY

Potato phosphorylase contains two moles of firmly bound organic phosphate/mole of enzyme which has been identified as pyridoxal-5-phosphate. Resolution of the enzyme and recombination with pyridoxal-5-phosphate were only partially successful because of denaturation of the enzyme at pH values below 5.5. In contrast to the corresponding mammalian enzymes, potato phosphorylase contains no serine phosphate. The specific phosphatase and the kinase for the serine phosphate groups of the mammalian enzyme had no effect on the potato enzyme.

Potato phosphorylase was found to contain 6 moles of sulfhydryl groups per mole of protein. The enzyme is inhibited by *p*-chloromercuribenzoate and this inhibition is only partially reversed by the addition of cysteine.

INTRODUCTION

The isolation of pyridoxal-5-P from muscle phosphorylase[1] and the demonstration that it functions as the prosthetic group of the enzyme[2] raised the question whether this vitamin was also an essential component of the plant phosphorylase system. Muscle phosphorylase *a* also contains serine phosphate[3], which can be split by a specific phosphatase, formerly called PR enzyme[4], and which can be rephosphorylated by a special kinase in the presence of ATP and Mg^{++}[5]. Here again it was of interest to compare the animal and plant phosphorylase systems. In a preliminary report[6] it has been shown that potato phosphorylase contains pyridoxal-5-P, whereas serine phosphate was found to be absent. The experimental evidence for these findings is reported in this paper.

MATERIALS AND METHODS

The preparation of potato phosphorylase and the measurement of its concentration and activity are described in the preceding paper[7]. PR enzyme[4] and phosphorylase *b* kinase[5] were prepared from rabbit skeletal muscle and the corresponding liver enzymes[8,9] from beef liver. A purified preparation of glutamic-aspartic transaminase from heart muscle was kindly supplied by Dr. S. VELICK.

Abbreviation used: pyridoxal-5-P, pyridoxal-5-phosphate.

Pyridoxamine phosphate was obtained from the California Foundation for Biochemical Research. Pyridoxal-5-P and other derivatives were purchased from Mann Research Laboratory, Inc. The amount of pyridoxal-5-P in solution was calculated from measurements in the Beckman spectrophotometer, based on the values for absorbancy established by PETERSON AND SOBER[10].

Phosphate determinations were carried out by the sensitive micromethod of CHEN *et al.*[11].

RESULTS

Identification of pyridoxal-5-P

According to PETERSON AND SOBER[10], pyridoxal-5-P in 0.1 N HCl shows a peak at 295 mμ ($E^{m}_{1\,\mathrm{cm}} = 6700$). In 0.1 N NaOH a new peak appears at 388 mμ ($E^{m}_{1\,\mathrm{cm}} = 6600$). A perchloric acid extract of the enzyme undergoes the same spectral changes when it is made alkaline. This is illustrated in Fig. 1. From the extinction at 295 and 388 mμ one calculates 1.9 and 2.1 moles of pyridoxal-5-P/mole of enzyme, respectively. The enzyme itself in 0.01 M phosphate buffer pH 6.8, when examined in sufficiently high concentration, shows a broad peak around 335 mμ; in 0.1 N NaOH this peak is replaced by a broad peak around 390 mμ (Fig. 2).

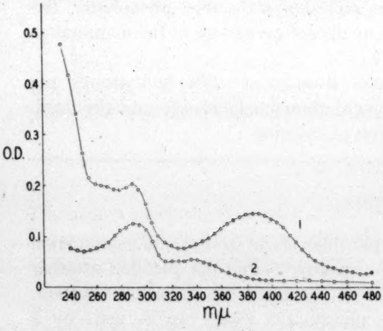

Fig. 1. Absorption spectra of the extract of 2.29 mg of potato phosphorylase. Curve 1, in 0.1 N NaOH; Curve 2, in 6 % perchloric acid.

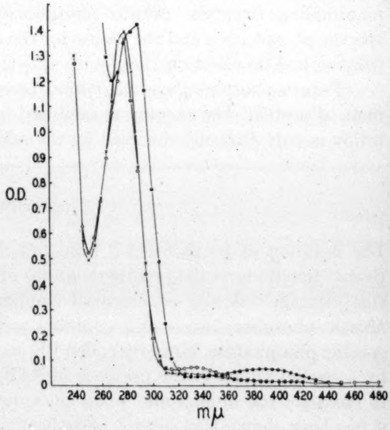

Fig. 2. Absorption spectra of potato phosphorylase, 1.055 mg/ml. ○, in 0.01 M potassium phosphate pH 6.8; ×, in 0.1 N HCl; ●, in 0.1 N NaOH.

Pyridoxal-5-P was isolated as the alcohol insoluble barium salt from a perchloric acid extract of the enzyme, as described by BARANOWSKI *et al.*[1]. After removal of barium ions with $SO_4^{=}$, the sample was used for paper electrophoresis, paper chromatography and enzymic tests with the glutamic-aspartic transaminase (a preparation consisting largely of apoenzyme). A part of the isolated material was also hydrolyzed for 6 h in 1 N HCl at 100°.

Paper electrophoresis was carried out in acetate buffer pH 5.1, ionic strength 0.05 in the Model R, Series D Spinco apparatus. A current of 3.5 mA was applied for 6 h. The bands appeared as blue fluorescence under u.v. light, which changed to yellow fluorescence on exposure to ammonia vapor. In a given experiment, an authentic sample of pyridoxal-5-P, a mixture of it with the isolated sample and the isolated sample alone all migrated the same distance. The acid hydrolysate of the isolated sample migrated at the same rate as pyridoxal.

Ascending paper chromatography on Whatman No. 1 filter paper was carried out at room temperature for 22 h in *n*-butanol–ethanol–glacial acetic acid–H_2O (5:3:0.5:1.5) solvent. The material isolated from the enzyme migrated at the same rate as pyridoxal-5-P, while the hydrolysate migrated like pyridoxal.

The activation of glutamic-aspartic transaminase, in an assay system similar to that of BARANOWSKI *et al.*[1] is shown in Fig. 3. Without the addition of pyridoxal-5-P, the reaction was slow. The material isolated from the enzyme, as well as heated potato phosphorylase, stimulated the transamination reaction to a similar extent as authentic pyridoxal-5-P.

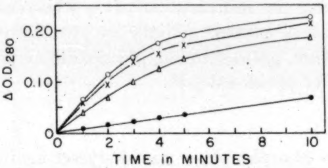

Fig. 3. Activation of glutamic-aspartic transaminase. ●, without the addition of pyridoxal-5-P; ○, with the addition of pyridoxal-5-P ($1 \cdot 10^{-5}$ *M*); ×, with the addition of the isolated material; △, with the addition of heated potato phosphorylase.

Resolution of enzyme

The apoenzyme of muscle phosphorylase *a* could be prepared in crystalline form[12]; it was completely inactive, but when pyridoxal-5-P was added, recombination took place and full activity was restored. The method of resolution consisted of lowering the pH of the enzyme solution with citrate buffer to about 4 in the presence of an excess of cysteine, followed by precipitation with ammonium sulfate. Muscle phosphorylase *b* could be resolved under milder conditions; incubation with 0.1 *M* Na glycerophosphate–0.08 *M* cysteine pH 6.0 at 0° for 12 h resulted in the complete resolution of the enzyme.

The difficulty encountered in attempts to resolve potato phosphorylase is that the enzyme is very unstable at pH values below 5.5 and that this pH is insufficient to liberate pyridoxal-5-P. When the enzyme was dialyzed against 0.1 *M* citrate–0.1 *M* cysteine, pH 5.5, no resolution occurred even in 24 h. The use of carbonyl reagents (semicarbazide, 0.1 *M* or hydroxylamine, 0.1 *M*) in place of cysteine was also unsuccessful.

Pyridoxal-5-P bound to the potato enzyme could be liberated below pH 4.5, but at the same time some unavoidable enzyme inactivation took place. One of the experiments is shown in Table I. To 0.5 ml of enzyme solution in an ice bath was added 0.2 ml of 0.1 *M* citrate–0.1 *M* cysteine, pH 4, 4.5 or 7.0. After 30 sec 3 ml of saturated ammonium sulfate and 1 drop of 0.1 *M* ammonia were added. The precipitate was washed once with alkaline ammonium sulfate solution and was dissolved in water. Addition of pyridoxal-5-P to the enzyme solution treated at pH 4

TABLE I

EFFECT OF PYRIDOXAL-5-PHOSPHATE ON ENZYMIC ACTIVITY

After the treatment (for details see text), the enzyme solutions were incubated for 30 min at 30° in $5 \cdot 10^{-2}$ *M* citrate buffer, pH 7 and $1.6 \cdot 10^{-4}$ *M* pyridoxal-5-phosphate before being assayed for enzymic activity.

Treatment	*Activity in units/mg protein*	
	Without P-5-P	*With P-5-P*
None	1820	1780
pH 4, 30 sec	0	153
pH 4.5, 30 sec	233	433
pH 7, 30 sec	1673	1673

restored some activity, but the recovery was less than 10%. Addition of 5′-AMP or cysteine did not improve the recovery. At pH 4.5 the enzyme was incompletely resolved, but a stimulation of enzyme activity by pyridoxal-5-P could nevertheless be demonstrated. Pyridoxine, pyridoxamine, pyridoxamine phosphate and pyridoxal could not be substituted for pyridoxal-5-P.

Phosphate content

A solution of potato phosphorylase was dialyzed against 0.1 *M* KCl–0.01 *M* $KHCO_3$, pH 8 for 3 days with 3 changes of buffer. A sample was ashed and analyzed according to the method of CHEN *et al.*[11]. The total phosphorus content was found to be 2 moles/mole of enzyme, that is, no organic phosphate except pyridoxal-5-P could be detected. The phosphorus content of the protein residue after precipitation with perchloric acid was analyzed, but no significant amount could be detected.

The absence of serine phosphate from potato phosphorylase is of interest. Unlike muscle phosphorylase *b* which is formed from phosphorylase *a* by the enzymic hydrolysis of serine phosphate and which requires 5′-AMP for activity, the potato enzyme is fully active in the absence of 5′-AMP. As might be expected, the specific phosphatase from muscle (PR enzyme) and from liver does not inactivate potato phosphorylase. There was also no inactivation during incubation with crude potato juice.

The question whether a serine group of potato phosphorylase can be phosphorylated by the muscle phosphorylase *b* kinase or kinase from beef liver in the presence of ATP and Mg^{++} has also been examined. An indirect coupling system was used to detect the formation of ADP by the phosphorylase *b* kinase reaction. This was based on the addition of phosphoenolpyruvate kinase and phosphoenolpyruvate to form pyruvate and ATP and on the addition of lactic dehydrogenase and DPNH to form lactate and DPN. The system worked satisfactorily with phosphorylase *b* or inactive liver phosphorylase as substrate. With potato phosphorylase no phosphorylation could be detected.

Sulfhydryl groups

The number of SH groups in potato phosphorylase was determined as follows. The enzyme solution was dialyzed extensively against 0.1 *M* Tris buffer pH 7.5 and 10 mg of the protein was titrated amperometrically with $1 \cdot 10^{-3}$ *M* $AgNO_3$ or $1 \cdot 10^{-3}$ *M*

$HgCl_2$. The values obtained were 5.69 and 6.0 moles of SH groups/mole of protein with $AgNO_3$ and 5.4 moles with $HgCl_2$. Titration of the SH groups with *p*-chloromercuribenzoate in 0.04 *M* glycerophosphate buffer pH 7.0 according to BOYER's[13] spectrophotometric method yielded a value of 6.8 moles/mole of enzyme. Muscle phosphorylase *a* contains 18 moles of sulfhydryl groups/mole of enzyme on the basis of amino acid composition and titration with *p*-chloromercuribenzoate[14]. The potato enzyme differs from the muscle enzyme in having fewer sulfhydryl groups per unit weight of protein.

Potato phosphorylase is not inhibited by iodoacetate ($1 \cdot 10^{-2}$ *M*). Heavy metals (Ag^+, Cu^{++}, Hg^{++}) are inhibitory, as shown in the preceding paper. Preincubation of the enzyme in glycerophosphate buffer pH 6.5 with a 100 fold excess of *p*-chloromercuribenzoate for 1 h at 30° gave a 60% inhibition and preincubation with a 10 fold excess for 5 h gave a nearly complete inhibition of enzyme activity. Contrary to what has been observed with muscle phosphorylase[14], it was not possible to restore completely the activity of the inhibited enzyme by the addition of an excess of cysteine. Experiments were also conducted at low temperature (2°), but here again there was only a partial reversal of the inhibited enzyme activity by cysteine (Fig. 4).

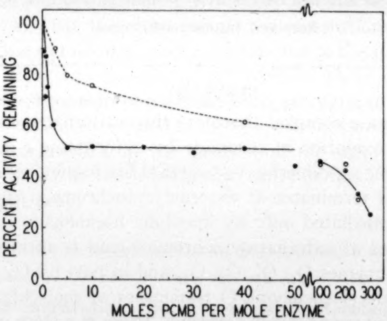

Fig. 4. Effect of *p*-chloromercuribenzoate (PCMB) on enzymic activity of potato phosphorylase. The enzyme, at a concentration of $1 \cdot 10^{-6}$ *M*, was incubated for 10 h at 2 ± 1° with PCMB in 0.04 *M* glycerophosphate buffer pH 6.5. The enzymic activity which remained was determined. ●—●, without further treatment; ○---○, after treatment with $5 \cdot 10^{-3}$ *M* cysteine for 3 h.

ACKNOWLEDGEMENTS

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STUDIES ON THE ELECTRON TRANSPORT SYSTEM

XXVI. SPECIFICITY OF COENZYME Q AND COENZYME Q DERIVATIVES

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SUMMARY

In succinic dehydrogenase complex there are three alternative pathways by which quinones mediate the oxidation of succinate by cytochrome *c* or oxygen. The first, mediated by menadione, 2,3-dimethoxy, 5-methyl benzoquinone and the short chain coenzyme Q analogues terminates at external cytochrome *c* and is antimycin insensitive. The second, mediated only by lipophilic homologues of coenzyme Q (Q_2, Q_3, Q_6, Q_{10}) terminates at external cytochrome *c* and is antimycin sensitive. The third, mediated by coenzymes Q_2, Q_3, Q_6, Q_{10} and in part by Q_0, Q_1, plastoquinone and heptyl and heptadecyl coenzyme Q terminates at molecular oxygen, involves bound cytochrome *c* and is antimycin sensitive. There is a close correlation between the effects of Q analogues on the succinoxidase activity of acetone extracted SDC and the effects on the succinoxidase activity of acetone extracted electron transport particle ETP. This suggests that the reconstituted succinoxidase system of succinic dehydrogenase complex closely resembles that of electron transport particle from which it was derived. Succinic cytochrome *c* reductase activity of succinic dehydrogenase complex, in principle, is a measure of the segment of the electron transfer chain which has been detached from the cytochrome *a* terminus of the chain. This segment by virtue of changes induced by the cleavage process shows a new pattern of quinone specificity. Only derivatives of 2,3-dimethoxy, 5-methyl benzoquinone with two or more isoprenoid units in the side chain at carbon 6 are able to serve as mediators in the antimycin sensitive oxidation of succinate by cytochrome *c*. The antimycin insensitive pathway involving cytochrome *c* *via* other quinones involves transfer of electrons from a site in the electron transfer chain prior to the locus for antimycin action.

Abbreviations: ETP, electron transport particle; SDC, succinic dehydrogenase complex.
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INTRODUCTION

In a previous communication of this series[1] it has been shown that coenzyme Q can be extracted by isooctane from particles with electron transport activity and that the loss of coenzyme is related to the loss of succinoxidase activity. The present communication deals with the specificity of coenzyme Q and homologues of coenzyme Q in the restoration of the activity of such solvent-extracted particles.

Cytochrome *c* reductase activity is generally assumed to be a measure of a segment of the same electron transfer chain which is involved in the oxidation of substrate by oxygen. The studies of GREEN AND MACKLER[2] in this laboratory were the first to cast doubt on this assumption. They showed that particles capable of oxidizing DPNH by oxygen did not interact with external cytochrome *c* and that the bound form of cytochrome *c* was not identical with the classical, water soluble form of cytochrome *c*. Later it was demonstrated that the ETP could be prepared in a form which has succinoxidase activity but no cytochrome *c* reductase activity[3]. The recognition of lipide cytochrome *c*[4,5] as the bound form of cytochrome *c* in ETP defined more precisely the basis for this divergence. Studies reported below on the reactivation of succinic cytochrome *c* reductase and succinoxidase activity by coenzyme Q show: (a) That the requirements for restoration of these two activities are different; (b) that cytochrome *c* reduced external to the particle cannot penetrate or penetrates too slowly to the site in the particle where oxidation by molecular oxygen in the terminal segment of the chain takes place; (c) that antimycin sensitivity of electron transport is closely related to the lipophilic nature of the coenzyme quinone which precedes cytochrome *c* in the chain; and (d) only 2,3-dimethoxy-5-methyl benzoquinones with one or more isoprenoid units in the 6 position can serve as a coenzyme in this pathway.

EXPERIMENTAL PROCEDURE

Enzyme preparations

Three enzyme preparations from beef-heart mitochondria were employed to determine the location of coenzyme Q in the electron transport system. The ETP which catalyzes the overall oxidation of succinate and DPNH by molecular oxygen without addition of cytochrome *c* was prepared by alkaline extraction of mitochondria[3]. This particle contains lipide cytochrome *c*, but does not interact with aqueous cytochrome *c* as determined by the fact that it shows very little cytochrome *c* reductase or reduced cytochrome *c* oxidase activity unless it is treated with reagents which remove lipide cytochrome *c*. The residue or insoluble fraction which is removed prior to the sedimentation of ETP has been referred to as the ETP-residue. This particle also catalyzes the oxidation of succinate and DPNH by molecular oxygen, but is inactive without the addition of cytochrome *c*. The electron transport system in this particle has been described as an "open" system in that it is readily capable of interaction with external cytochrome *c* and without any pretreatment shows succinic and DPNH cytochrome *c* reductase activity as well as reduced cytochrome *c* oxidase activity. The ETP-residue obtained during separation of ETP from mitochondria by alkaline treatment was used in these experiments.

When ETP is treated with amyl alcohol or deoxycholate a red particle is formed which has succinic cytochrome *c* reductase activity. This type of particle is designated as SDC[6,7]. The SDC particles prepared by either method are similar in their basic properties, but differ in some details. In the experiments reported here SDC was prepared by deoxycholate treatment of ETP. This preparation which has previously been referred to as the "red fraction"[6] contains a small amount of cytochrome *a* and when supplemented with cytochrome *c* it catalyzes the oxidation of succinate by oxygen. We have used this type of preparation to compare the effect of solvent extraction on succinic cytochrome *c* reductase and succinoxidase activity.

Extraction procedures

Two extraction procedures were used to remove coenzyme Q from the particles, and a number of polar and non-polar solvents were tried. The first procedure (wet extraction) involved extraction of the particles suspended in 0.25 *M* sucrose solution while the second involved extraction of dry lyophilized enzyme preparations with the solvent (dry extraction). Following extraction, as much solvent as possible must be removed from the particles to prevent solvent inhibition of electron transport. We find that exposure of the suspension to a vacuum for 0.5 h at room temperature, followed by centrifugation of the extracted particles diluted in sucrose solution, removes sufficient solvent to rule out solvent inhibition effects.

In the wet extraction, the sucrose suspension of the enzyme preparation (15–20 mg protein/ml) was shaken in a glass stoppered tube with four volumes of the solvent at room temperature (25°). Shaking was carried out on a reciprocal shaking machine at 300 strokes/min with stroke length of 30 cm. After the necessary time of shaking, the mixture was spun in a clinical centrifuge and separated into three phases. The particulate phase was then separated, exposed to vacuum for about 30 min and resuspended in fresh sucrose solution. The suspension was then homogenized and centrifuged at 40,000 RPM for 30 min. The sediment was taken up in sucrose solution and the homogenized suspension was then used directly.

The details of extraction of ETP with isooctane have been described previously[1,8]. We have followed essentially the same method except that Versene and acetate buffer were omitted during extraction of SDC since activity is irreversibly lost when these reagents are present.

In the dry extraction, 150 mg of enzyme powder were mixed with 15 ml solvent, and the suspension was shaken for 30 to 90 min on the reciprocal shaker as described above. The particulate material was then separated by centrifugation and the solvent decanted. Excess solvent was removed at room temperature under vacuum. The particles were washed in sucrose by high speed centrifugation and resuspended in sucrose for activity determination. The lyophilized powder of ETP or SDC was resuspended in a requisite amount of distilled water to achieve the sucrose concentration of the original suspension prior to lyophilization. These suspensions served as controls for the effect of solvent extractions.

Homologues and analogues of coenzyme Q

Coenzyme Q_{10} was isolated from heart muscle by the method of CRANE *et al.*[9] and coenzyme Q_6 by a modification* of the same procedure followed for coenzyme

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* Coenzyme Q_6 was prepared by saponification of bakers yeast and isolation from the non-saponifiable fraction by the method used for coenzyme Q_{10} from beef heart. Preliminary crystallization was effected from ethanol–methanol (1:1, v/v) followed by crystallization from ethanol at −15°.

Q_{10}. CRANE[10] has described the method of isolation of plastoquinone (Q_{254}) from alfalfa meal. The following synthetic homologues and analogues of coenzyme Q were made available to us through the courtesy of Dr. K. FOLKERS of the Merck Sharp and Dohme Laboratories in Rahway, New Jersey: coenzymes Q_0* (aurantiogliocladin), Q_1, Q_2, Q_3; heptyl, phytyl and heptadecyl coenzyme Q; eicosahydrocoenzyme Q_{10}; coenzyme Q_{10} diacetate; 2,3-dimethoxy, 5-methylbenzoquinone, and various other substituted benzoquinones. The methods used for the synthesis of some of these compounds are described by WOLF *et al.*[11].

Chemicals

Cytochrome *c* was obtained from Sigma Chemical Laboratories; menadione, Vitamin K_2 from mycobacteria was a generous gift of Dr. H. NOLL, University of Pittsburgh. Synthetic vitamin K_2 derivatives containing side chains with varying number of isoprenoid units were obtained through the courtesy of Dr. A. BRODIE, Harvard University, and Prof. L. FIESER of Harvard University kindly provided several naphthoquinones. The acetone used for extraction purposes was an analytical grade supplied by Fischer Scientific Co.

Methods of assay

The assay of enzyme activity was carried out essentially by the methods which we have previously described[3].

Succinoxidase activity was determined manometrically in the Warburg apparatus. The reaction mixture contained 100 μmoles of succinate, 40 μmoles of potassium phosphate pH 7.4, 1.3 μmoles of sodium Versenate, and approx. 1 mg of enzyme in a volume of 1.3 ml; 0.2 ml of 6 *N* KOH was added to the center well. Quinones and other lipid supplements were added in 0.05 ml of ethanol and ethanol was added to the control flasks.

DPNH oxidase activity was determined spectrophotometrically. The reaction mixture contained 0.08 mg of DPNH and 80 μ*M* of potassium phosphate pH 7.4 in a total volume of 1.0 ml, sufficient enzyme was added to give a change in absorbancy from 0.02 to 0.10/min.

Succinate cytochrome *c* reductase was determined spectrophotometrically by decrease in O.D. at 550 mμ. The reaction mixture contained 80 μ*M* of potassium phosphate pH 7.4, 100 μ*M* of potassium succinate pH 7.4, 0.4 μ*M* of potassium cyanide and 0.2 mg of cytochrome *c* in a total volume of 1.0 ml. Sufficient enzyme was used to give a change in absorbancy of from 0.02 to 0.1/min.

RESULTS

Restoration of succinic-cytochrome c *reductase activity of SDC*

When the lyophilized powders of the various particles are extracted for 30 min or more with acetone, some 80% of the coenzyme Q content is removed and the addition of coenzyme Q alone suffices to restore cytochrome *c* reductase activity. Both coenzyme Q and cytochrome *c* are required for restoration of succinoxidase activity. When the extraction period is extended beyond 30 min an additional lipide factor is required for succinoxidase activity. This factor has been mentioned previously[12] and will be discussed more extensively in a later communication. Suspensions prepared from lyophilized SDC which has been extracted with acetone for 90 min no longer catalyze the reduction of cytochrome *c* by succinate. This activity can be restored by homologues and analogues of coenzyme Q to the degree indicated in Table I. The positive results obtained with heptyl and phytyl coenzyme Q and eicosahydrocoenzyme Q_{10} suggest that the degree of unsaturation in the side chain is not critical for activity although it has a definite influence.

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* The subscript in each case indicates the number of monounsaturated isoprenoid units in the side chain at position 6. Other substitutions at position 6 are described by naming the side chain e.g. heptyl coenzyme Q with a heptyl side chain replacing the isoprenoid side chain.

TABLE I

RESTORATION OF SUCCINIC CYTOCHROME *c* REDUCTASE AND SUCCINOXIDASE ACTIVITIES OF SDC*
BY COENZYME Q AND RELATED QUINONES

*Compound added****	*Mg added*	*Succinic cytochrome c reductase***		*Succinoxidase*	
		No antimycin	*Antimycin§ added*	*No antimycin*	*Antimycin added*
None		0.00	0.00	0.16	0.00
Q_0	0.05	0.22	0.24	0.32	0.09
Q_1	0.05	0.31	0.25	0.62	0.05
Q_2	0.05	0.37	0.09	0.72	0.00
Q_3	0.05	0.42	0.00	0.78	0.00
Q_6	0.05	0.55	0.00	0.68	0.00
Q_{10}	0.10	0.61	0.00	0.50	0.00
Heptyl Q	0.05	0.31	0.31	0.29	0.00
Heptadecyl Q	0.05	0.09	0.00	0.37	0.00
Phytyl Q	0.05	0.35	0.05	0.44	0.00
2,3-dimethoxy, 5-methyl benzoquinone	0.05	0.15	0.18	0.00	0.00
Menadione	0.05	0.18	0.18	0.36	0.13
Vitamin K_1	0.05	0.00	0.00	0.00	0.00
2,3-dimethyl-1,4-naphthoquinone	0.05	0.00	0.00	0.00	0.00
Plastoquinone	0.10	0.00	0.00	0.24	0.00

* Lyophilized SDC (specific activity before extraction: cytochrome *c* reductase, 0.6; succinoxidase, 0.80) extracted with acetone for 90 min and processed as described.

** All activities in presence of added cytochrome *c*; concentration of supplement/mg protein: cytochrome *c*, 1.0 mg; Q_{10} and Q_{254}, 0.1 mg; other compounds 0.05 mg each.

*** All compounds reported at levels which gave optimum activity; range of concentration tested 10–50 μg; the inactive compounds were tested in the range 0.01–0.10 mg, numericals suffixed to Q denote the number of isoprenoid units in the side chain at 6 position as homologue of coenzyme Q. Plastoquinone (Q_{254}) is a quinone common to green plants and was prepared from alfalfa.

§ Antimycin, wherever inhibitory, was active even at 1 μg/100 μg enzyme protein, range tested 1–10 μg/100 μg protein.

The activity induced by the higher coenzyme Q homologues (Q_3–Q_{10}) and by heptadecyl coenzyme Q is completely antimycin sensitive whereas that of the following homologues and analogues is sensitive only in part: Coenzyme Q_1 (20 % sensitive); coenzyme Q_2 (75 % sensitive); and phytyl coenzyme Q (85 % sensitive). The activity induced by coenzyme Q_0, menadione and 2,3-dimethoxy, 5-methylbenzoquinone is completely insensitive to antimycin.

Table I also shows that coenzyme Q cannot be replaced by plastoquinone (Q_{251}) and vitamin K_1; α-tocopherol, beef serum albumin, mitochondrial carotene, phospholipides and cholesterol do not restore activity. The diacetate of reduced coenzyme Q_{10} is also inactive whereas the reduced form of coenzyme Q_{10} has the same activity as the oxidized form. The Q lipoprotein[13] which contains coenzyme Q in an available form is fully as active as free coenzyme Q_{10}. The amount of Q required for maximal restoration is, however, less when the coenzyme is added in the form of the lipoprotein. The acetone extract of lyophilized SDC after removal of material insoluble in cold alcohol has comparable activity to that of coenzyme Q_{10} when compared at the same concentration level of coenzyme Q_{10}. The crude acetone extract contains inhibitory substances which obscure the restoration effect.

The increase in cytochrome *c* reductase activity is a function of coenzyme Q_{10} concentration. When the ratio of SDC to coenzyme Q_{10} (mg protein/μg of coenzyme) is 1.7, the system is half saturated. At saturation the ratio is 0.8.

The residue fraction which is a byproduct in the isolation of ETP[3] (referred to as ETP residue) has considerable succinic-cytochrome *c* reductase activity. Since this particle has an open electron transfer chain compared to the closed chain of ETP[2,3] it was of interest to determine the requirements for the cytochrome *c* reductase activity of the ETP residue. The results in Table II show that the activity is largely lost following extraction of the lyophilized particles with acetone, and that coenzyme Q can restore the lost activity, whereas α-tocopherol, vitamin K_1 or albumin are inactive in this respect. The pattern for the ETP residue closely resembles that for SDC.

TABLE II

RESTORATION OF CYTOCHROME *c* REDUCTASE ACTIVITY OF ETP-RESIDUE

State of particle	*Additions*	*Succinic cytochrome c reductase activity μM succinate/min/mg proiein*
Original (fresh)	None	0.21
Lyophilized	None	0.26
Lyophilized	CoQ	0.25
Extracted*	None	0.06
Extracted	CoQ, 12 μg	0.16
Extracted	CoQ, 25 μg	0.29
Extracted	Acetone extract (20 μg CoQ)	0.28
Extracted	*d*-*α*-tocopherol, 25 μg	0.06
Extracted	Vitamin K_1, 25 μg	0.05
Extracted	Beef serum albumin, 0.1–1.0 mg	0.08

* The ETP-residue was extracted for 60 min with acetone and processed as described in the text.

Restoration of succinoxidase activity of SDC

During acetone extraction of SDC, succinic-cytochrome *c* reductase activity is the first to disappear and then later succinoxidase activity. Providing the period of extraction is not excessive only two components are required for restoration of succinoxidase activity viz. coenzyme Q and cytochrome *c*. The various homologues and analogues of coenzyme Q which can restore succinoxidase activity are listed in Table I. These include some homologues of coenzyme Q from Q_0 to Q_{10}, heptyl, heptadecyl and phytyl coenzyme Q. Coenzyme Q_3 is the most active of the compounds tested. The activity induced by all but two of the compounds mentioned is antimycin sensitive. The activity in presence of aurantiogliocladin and coenzyme Q_1 is respectively 72 % and 92 % inhibited by antimycin.

Heptyl coenzyme Q which induces an antimycin insensitive cytochrome *c* reductase activity, by contrast gives rise to succinoxidase activity which is completely antimycin sensitive. The menadione-induced oxidase activity is atypical in two respects: (a) It is insensitive to cyanide; and (b) does not require the mediation of cytochrome *c* (F. L. CRANE AND K. S. AMBE, unpublished data). Activity induced by coenzyme Q derivatives is both cyanide-sensitive and dependent upon the presence of externally added cytochrome *c*. Plastoquinone is inactive in the reductase system but shows appreciable activity in the oxidase system when cytochrome *c* is present. 2,3-dimethoxy, 5-methyl benzoquinone not only is inactive in the oxidase assay but it also inhibits the catalytic effect of coenzyme Q_{10}.

Restoration of succinoxidase activity of ETP

The degree of specificity of coenzyme Q compounds in restoring the succinoxidase activity of acetone extracted ETP is higher than in the restoration of the activity of acetone extracted SDC. Both aurantiogliocladin (Q_0) and menadione induce succinoxidase activity in the extracted SDC which to a limited extent is antimycin insensitive (*cf.* Table I and III). When added to extracted ETP, however, these compounds restore little activity and whatever activity reappears is entirely antimycin sensitive. In other respects the specificity of coenzyme Q compounds for restoration of succinoxidase activity in acetone extracted ETP is the same as observed in SDC, α-tocopherol, tetra-methoxy coenzyme Q_{10}, diacetyl dihydro coenzyme Q_{10}, vitamin K_1, vitamin K_2 from mycobacteria and synthetic forms of vitamin K_2 with isoprenoid side chains containing two to four isoprene residues respectively, as well as beef serum

TABLE III

RESTORATION OF SUCCINOXIDASE ACTIVITY OF ETP BY HOMOLOGUES AND ANALOGUES OF COENZYME Q

State of particle	*Coenzyme added*	*Succinoxidase activity** *µM succinate/ min/mg protein*
Lyophilized	None	0.53
Extracted*	None	0.00
Extracted	Q_0	0.11
Extracted	Q_1	0.52
Extracted	Q_2	0.52
Extracted	Q_3	0.50
Extracted	Q_{10}	0.49
Extracted	Phytyl Q	0.40
Extracted	Heptyl Q	0.40
Extracted	Heptadecyl Q	0.22
Extracted	Menadione	0.03

* All activities were measured in presence of 1.0 mg cytochrome *c*. Concentration of supplements giving optimum activity added per assay per mg protein: Coenzyme Q_{10} and heptadecyl Q, 0.1 mg, all other forms of coenzyme Q and menadione 0.05 mg. There was no activity in the presence of antimycin.

** Lyophilized ETP was extracted with acetone for 30 min.

albumin are inactive[*]. Only derivatives of 2,3-dimethoxy, 5-methyl benzoquinone with a fatty side chain in the 6 position are capable of restoring succinoxidase activity, and this restored activity is antimycin sensitive.

Succinoxidase versus *DPNH oxidase activity of ETP*

DPNH oxidase activity is destroyed by extraction of particles with acetone and cannot be restored by addition of cytochrome *c* or coenzyme Q or both (*cf.* Table IV). However, addition of the acetone extract back to the particle along with cytochrome *c*, results in a slight restoration of DPNH-oxidase and a very marked restoration of succinoxidase activity. In the case of ETP extracted with isooctane, cytochrome *c* alone could replace the requirements of coenzyme Q and phospholipid in reactivating succinoxidase[1]. Only under very drastic conditions of extraction, are all three components necessary for restoration. These phenomena could be explained in terms of a bypass of electrons from the succinic to the DPNH chain and vice versa as discussed previously[1].

TABLE IV

SUCCINOXIDASE VS DPNH-OXIDASE ACTIVITY OF ETP

State of particle	*Additions*	*Succinoxidase activity*	*DPNH-oxidase activity*
		μM substrate oxidized/min × mg protein	
Lyophilized	None	0.51	0.68
Lyophilized	Cytochrome *c* + CoQ + acetone extract	0.48	0.52
Extracted[*]	None	0.00	0.00
Extracted	Cytochrome *c*	0.00	0.00
Extracted	Cytochrome *c* + CoQ	0.18	0.00
Extracted	Cytochrome *c* + CoQ + acetone extract	0.42	0.04
Extracted	Cytochrome *c* + acetone extract	0.40	0.05
Extracted	Acetone extract	0.15	0.00

[*] Lyophilized powder of ETP extracted for 30 min with acetone. Concentrations of supplements in both assays per mg enzyme protein: Cytochrome *c*, 0.66 mg; coenzyme Q (CoQ), 0.066 mg; acetone extract, equivalent of the original enzyme.

DISCUSSION

There have been several recent reports on the restoration of the cytochrome *c* reductase activity of isooctane-extracted particles by various lipide materials[14–17]. Apart from the fact, that these effects were not specific there was in none of these instances any evidence that the loss in activity of the particles sustained by the solvent extraction, could be correlated with the extraction of the component from the particle. In the present studies[1] the loss of enzymic activity could be correlated with extraction of coenzyme Q and the concentration of the coenzyme required for reactivation of the particle was of the same catalytic order of magnitude as the concentration of coenzyme in the extract.

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[*] In alternate experiments with isooctane extracted ETP, essentially the same specificity was observed in the restoration of succinoxidase. Various benzoquinone derivatives with the following substituents were tested and found inactive in these experiments: 2,3-dimethoxy; 2,5-dimethoxy, 3-pentadecyl; 2,5-dimethoxy, 3,6-dipentadecyl; 2,5-dimethoxy, 3,6-diphytyl; 2,5-dihydroxy, 3-pentadecyl; 2,5-dihydroxy, 3,6-dipentadecyl; 2,6-dimethoxy. 2,5-dimethoxy, phytyl benzoquinone, however, showed some trace activity.

NASON *et al.*[17] have presented evidence that α-tocopherol is required for restoration of the succinic-cytochrome *c* reductase activity of isooctane extracted particles. These authors were in fact the originators of the immensely useful technique of wet solvent extraction of enzyme solutions and particle suspensions. POLLARD AND BIERI[16] interpret the effect of α-tocopherol in terms of the unspecific reversal of the inhibitory effects referable to residual traces of solvent in the particle suspension. DONALDSON *et al.*[17] were unable to correlate loss of activity with extraction of α-tocopherol.

WEBER, GLOOR AND WISS[14] and REDFEARN AND PUMPHREY[15] found that coenzyme Q_{10} was only one of a group of several isoprenoid compounds which could restore the succinic-cytochrome *c* reductase activity of isooctane-extracted particles. Under the extraction conditions used by these authors (extraction in the cold) there is minimal extraction of coenzyme Q_{10}. The action of coenzyme Q and the other isoprenoid compounds in such a system is often a reversal of solvent inhibition[15] and is not specific. Obviously, coenzyme effects are demonstrable only with particles from which the bulk of coenzyme Q has been extracted.

Under the conditions described in these studies, the specificity of coenzyme Q is very pronounced, especially in the succinoxidase system. α-Tocopherol, vitamin K_1, vitamin K_2 from mycobacteria and synthetic forms of vitamin K_2 with isoprenoid side chains containing 2–6 isoprene residues were all found to be inactive. Only derivatives of 2,3-dimethoxy, 5-methyl benzoquinone with a fatty side chain in the 6 position are capable of restoring antimycin sensitive succinoxidase activity which is mediated by cytochrome *c*. Aurantiogliocladin (coenzyme Q_0) and menadione show some activity, a minor fraction of which is antimycin insensitive only in the case of SDC.

A study of the restoration of succinic cytochrome *c* reductase and succinoxidase activity by derivatives of coenzyme Q reveals that activity in the two systems is not necessarily restored to the same extent by a given quinone. Unless cytochrome *c* is reduced at the antimycin sensitive site it does not contribute to succinoxidase activity. Thus, menadione and aurantiogliocladin restore in part the succinic cytochrome *c* reductase activity of acetone extracted SDC. But this activity unlike that induced by lipophilic forms of coenzyme Q is antimycin insensitive. It would appear that the pathway between succinate and cytochrome *c*, mediated by menadione and aurantiogliocladin, skips the antimycin-sensitive site. Probably cytochrome *c* reacts directly with the reduced forms of these two quinones. There is an analogous direct reaction between reduced coenzyme Q and cytochrome *c* which according to HATEFI[18] takes place in the lipoprotein in which coenzyme Q is localized. This reaction is also antimycin insensitive. However, this artificial interaction does not go on in a particle to which both coenzyme Q and cytochrome *c* have been added as shown by the complete antimycin sensitivity of the process. This result suggests that coenzyme Q is anchored to sites or components in the lipoprotein which either do not react with other quinones or which are inaccessible to them. It also appears that the portion of the externally added cytochrome *c* which functions in oxidase activity is restricted in its operation as if it were reincorporated within a "closed" system.

SDC can also catalyze an antimycin insensitive reduction of cytochrome *c* by coenzyme Q derivatives with short isoprenoid or alkane substituents in the 6 position of the benzenoid nucleus as well as by other quinones such as menadione and 2,3-dimethoxy, 5-methyl benzoquinone. RAMASARMA AND LESTER[19] have previously observed that many quinones of diverse structure are reduced by succinate in the presence of mitochondria. It has been shown by MAHLER *et al.*[20] that many reduced quinones are able to interact non-enzymically with ferri cytochrome *c*. Although the antimycin insensitive pathway is obviously artifactual, it is not without interest in view of the specificity of the particle with respect to the nature of the quinone which can participate in the interaction with cytochrome *c*. Benzoquinone, paraxylobenzoquinone and the fully substituted naphthoquinones are ineffective as catalysts for cytochrome *c* reductase.

The possibility of restoring antimycin sensitive cytochrome *c* reductase and oxidase activity with succinate as substrate is restricted specifically to forms of coenzyme Q with long side chains and is accomplished most efficiently by compounds which possess an unsaturated isoprenoid side chain containing more than two isoprene residues.

The low activity of coenzyme Q_{10} in the assay for succinoxidase activity may be surprising. However, as discussed previously[12] acetone-extracted particles show some requirement for a lipid component NL II, and this compound is essential for the activity only of coenzyme Q analogues which possess a side chain of more than two isoprenoid units. In presence of NL II, coenzyme Q_{10} is as active as coenzymes Q_2 or Q_3. By contrast coenzyme Q_{10} is maximally active without added NL II in the restoration of the cytochrome *c* reductase activity of the same particle. The heptadecyl derivative of coenzyme Q as well as plastoquinone are atypical in that the restoration of cytochrome *c* reductase activity which they induce is negligible compared to the restoration of oxidase activity.

In the case of SDC, the particle which was most extensively studied, the following pattern has emerged from the present work: SDC has three pathways involving cytochrome *c* (or oxygen) which respond to the addition of quinones. The first is the antimycin insensitive pathway to external cytochrome *c* induced by addition of menadione, 2,3-dimethoxy, 5-methyl benzoquinone and short chain coenzyme Q analogues. The second is the antimycin sensitive pathway that can be induced only by homologues of coenzyme Q_{10} (ranging from Q_{10} to Q_2). The third is the antimycin sensitive pathway to molecular oxygen which involves bound cytochrome *c* and which can operate only in the presence of members of the coenzyme Q family of compounds of analogues of coenzyme Q (heptyl and heptadecyl coenzyme Q), and plastoquinone.

The possibility has to be entertained that particles like SDC which are derived by deoxycholate fragmentation of ETP may have artifactual activities and that the requirement of any particular activity like cytochrome *c* reductase activity for coenzyme Q, may not necessarily reflect a coenzymic role for the quinone in overall electron transport. But the fact that particles like the ETP residue which are formed from mitochondria by a relatively mild procedure also show exactly the same requirement for reductase activity, lends support to the hypothesis of a true coenzymic role for the mitochondrial quinone.

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FACTORS AFFECTING THE SWELLING OF KIDNEY CORTEX SLICES AT 0°

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SUMMARY

Factors affecting swelling of slices of rabbit kidney cortex at 0° in isotonic salines containing Na^+ as the main cation and Cl^- as the bulk anion were examined.

1. Tissue hydration and chlorides increase with increasing concentration of K^+ in the leaching medium. A similar swelling effect was shown for Rb^+, but not for Li^+.

2. A linear relationship was shown to exist between the apparent membrane potential, calculated from the Donnan ratio of potassium, and the logarithm of the external potassium concentration.

3. An inverse relationship was demonstrated between tissue water and the apparent Donnan ratios of chloride and potassium: as compared with controls leached at 0° in standard saline $\left(\frac{[Cl^-]_o}{[Cl^-]_i} = 1.495 \pm 0.037; \frac{[K^+]_i}{[K^+]_o} = 8.86 \pm 0.35\right)$ factors increasing tissue swelling, *e.g.*, 0.2–0.6 *mM* $HgCl_2$, 0.7–1.4 *mM* Esidrone, 2–149 mEqu K^+, 67 and 134 mEqu Rb^+, decreased $\frac{[Cl^-]_o}{[Cl^-]_i}$ and $\frac{[K^+]_i}{[K^+]_o}$, whilst 1–6 m*M* Ca^{2+}, Ba^{2+}, Sr^{2+} and Mn^{2+} decreased tissue swelling and also increased these ratios.

4. The relationships found between tissue water and the apparent Donnan ratios of chloride and potassium correspond to a derived relationship of the osmotic gradient and the ratio $\frac{[Cl^-]_o}{[Cl^-]_i}$ for a double Donnan system.

INTRODUCTION

It is now well established that animal tissues swell on immersion at 0° in isotonic sodium chloride or in isotonic salines of the KREBS-RINGER type (*e.g.*, ROBINSON[1], MUDGE[2], AEBI[3], WHITTAM AND DAVIES[4]) and reach within about 2 h an apparent equilibrium between the tissue water and electrolytes and the medium; the term "balanced state" of the tissue has been suggested by CONWAY[5] to denote this apparent equilibrium. The mechanism of the swelling process has been the subject of a number of investigations. Evidence has been presented suggesting that the swelling of *e.g.* kidney cortex slices at 0° may be accounted for by a double DONNAN system (LEAF[6]), although osmotic changes due to the breakdown of intracellular macromolecules into smaller moieties may also contribute to the swelling process (CONWAY, GEOGHEGAN

AND McCORMACK[7]); in both these investigations it was assumed that the active transport of solutes (and water) at 0° was negligible and that the balanced state of the tissue represented an equilibrium, as opposed to a steady state. However, a basically different view was advanced by MAIZELS AND REMINGTON[8]. These authors pointed out that the water and electrolyte content of the tissue after leaching at 0° could hardly represent an equilibrium state since, due to the presence of an indiffusible anion in a Donnan system, there would be a tendency of the water and electrolytes to continuously enter the cell with the eventual result that the cells would burst. The view was therefore put forward that at 0–2° active transport of sodium (and water) counteracted the influx of sodium and water due to Donnan forces. The apparent equilibrium between the tissue solutes and the medium after leaching at 0° was thus considered to be the result of a new steady state of the cellular components.

It has been shown elsewhere (KLEINZELLER AND CORT[9, 10]) that 0.2–1.4 *mM* mercurial preparations, both inorganic and organic, and also 0.6 m*M* Cu^{2+} and 1.2 *mM* Ag^+, considerably increase the swelling of kidney cortex slices during leaching for 2.5 h at 0° in isotonic sodium chloride, increase the fluxes of sodium and potassium and of urea at the balanced state of the tissue components, and decrease the apparent Donnan ratios of potassium and chloride ions. On the other hand, 1–6 *mM* Ca^{2+}, and also ions of some other alkaline earths were found to have the opposite effect, *i.e.* decreasing the swelling of the tissue and the fluxes of the cations and of urea at the balanced state, and also increasing the apparent Donnan ratios of potassium and chloride ions (KLEINZELLER AND CORT[11]). A detailed investigation of the relationship between the tissue electrolytes and the water after leaching kidney cortex slices at 0°, and the effect of various agents thereon, appeared therefore desirable, especially since it was known from the now classical work of BOYLE AND CONWAY[12] that potassium greatly affected the swelling of some tissues, *e.g.* muscle.

It will be shown here that potassium and rubidium ions increase the swelling of kidney cortex slices at 0° in a similar way to that in muscle. Furthermore, evidence will be presented for a relationship between the apparent Donnan ratios of potassium and chloride, and the water content of the tissue. The simplest explanation of this observation appears to be a Donnan system with some agents, which affect tissue swelling, directly influencing the charge of the cellular membrane.

A preliminary account of these results has been presented by KLEINZELLER[13].

EXPERIMENTAL

The experimental methods used here have been described in detail elsewhere (KLEINZELLER AND CORT[11]) and are only summarized here. Unless otherwise stated, kidney cortex slices of healthy rabbits were leached 2.5 h in a saline solution of the following composition (in mEqu/l redistilled water): 142.5 Na^+; 5.1 K^+; 7.6 Li^+; 148 Cl^-; 7.2 HPO_4^{2-}; pH 7.4, or in salines with various cations replacing Li. The salines were cooled with crushed ice, the cut slices then transfered into these, and the salines with the slices maintained at 0° in an ice-chest, with occasional shaking. The following tissue components were estimated: Tissue water, Na and K (by flame photometry) and Cl^- potentiometrically by the method of SANDERSON[14]. Results, representing the mean of at least two analyses, are expressed in ml H_2O and mEqu of ions per

100 g dry tissue solids (DS). For the calculation of the apparent intracellular concentrations of potassium and chloride, the value of the inulin extracellular space of 0.20 ml/g tissue was taken for slices leached in standard saline or in the presence of mercurials, whilst for slices leached in the presence of alkaline earths the slightly higher value of 0.23 ml/g was used (Kleinzeller and Cort[10, 11]). Molal concentrations (mEqu/kg H_2O) of ions are denoted by square brackets, indices *i* and *o* refering to the intracellular and extracellular spaces, respectively.

As an organic mercurial, Esidrone (*i.e.*, theophyllino–mercuri–monooxypropylamido–sodiumquinolate, Ciba, Basel, Switzerland) was used.

RESULTS

The effect of some metabolic inhibitors on the swelling of kidney cortex slices at 0°

The effect of some metabolic inhibitors on the swelling of kidney cortex slices at 0° was examined in order to test whether any active transport could be demonstrated at this temperature, as suggested by Maizels and Remington[8]; if this were so, substances such as dinitrophenol (DNP) or iodoacetate might be expected to inhibit such a transport process and bring about an increased swelling of the slices.

It may be sufficient to relate that these experiments were clearly negative; DNP at concentrations up to 0.5 *mM* did not affect the swelling; it has been shown elsewhere (Kleinzeller and Cort[10]) that 1–2 *mM* iodoacetate and also a number of other agents known to block thiol groups were similarly found to be without effect on the swelling. Unless evidence can be provided that DNP and other inhibitors do not penetrate into the cells at 0°, these negative results are suggestive of the view that no active transport of sodium (and water) takes place at 0° in kidney cortex slices.

The effect of alkaline cations on the swelling of kidney cortex slices at 0°

Aebi[3] has shown that equivalent replacement of sodium by potassium in the leaching saline or incubating medium at 37° produced an increased swelling of kidney cortex slices; no comments were offered as to the mechanism of this swelling effects of potassium.

The findings of Aebi were confirmed and extended also for tissue chloride. The results of a representative experiment, presented in Fig. 1, show that increasing concentrations of K^+ in an isotonic saline bring about an increase of tissue water, potassium and chloride, and a decrease of tissue sodium. These results are in agreement with those obtained by Boyle and Conway[12] for muscle, Kleinzeller and Rybová[15] for brain cortex slices, and by Kleinzeller, Kolínská and Folbergrová[16] for pigeon pancreas slices. It thus may be assumed that the swelling effect of potassium in these tissues is brought about by the same mechanism, *i.e.* by a Donnan system as described by Boyle and Conway.

If from these results the apparent $[K^+]_i$ is calculated and the apparent membrane potential φ $\left(\varphi = 54 \log \frac{[K^+]_i}{[K^+]_o}\right)$ then plotted against log $[K^+]_o$, a straight line is obtained, as shown in Fig. 2. Such a result is in agreement with the view that the cellular membrane of kidney cortex cells is freely permeable to potassium. The apparent Donnan ratio of chloride decreased in this experiment from the value of 1.53 (no potassium in the medium) to 1.19 (149 mEqu K^+/kg medium), corresponding to a change of apparent membrane potential from 10 to 4.17 mV.

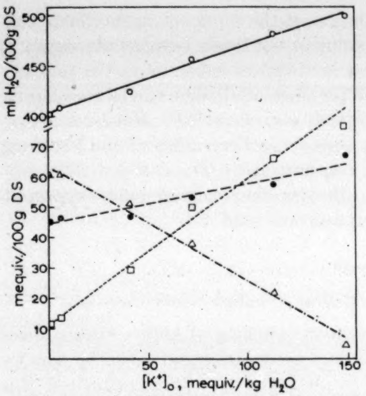


Fig. 1. Effect of varying concentrations of potassium in the leaching medium on tissue water and electrolytes: kidney cortex slices leached 2.5 h at 0° in 0.154 *M* NaCl, buffered with 0.05 vol. 0.1 *M* Na-phosphate buffer, pH 7.4, or in salines with 0.154 *M* KCl equivalently replacing NaCl. ○—○ H_2O (ml/100 g DS); △-·-·-△ Na^+, □----□ K^+, ●—● Cl^-, all in mEqu/100 g DS.

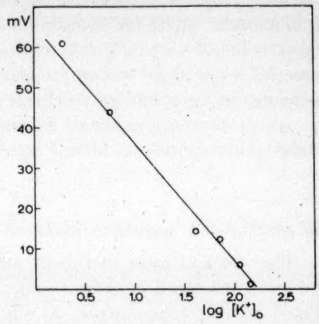


Fig. 2. Relationship of apparent membrane potential as a function of varying concentrations of K^+ in the leaching medium. For conditions of experiment, see Fig. 1. The apparent membrane potential

$$\varphi = 54 \log \frac{[K+]_i}{[K+]_o}, \text{ in mV.}$$

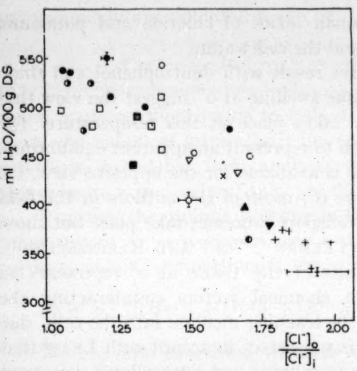
The effect of potassium is not specific for this ion, the same effect being shown by Rb^+, but not by Li^+ (Table I). This result appears to be in agreement with the view of CONWAY[5] that the difference in the behaviour of these two cations is a reflection of the diameter of their hydrated form, hydrated Rb^+ being considerably smaller than Li^+ and therefore freely diffusible through the cellular membrane.

TABLE I

EFFECT OF VARIOUS ALKALINE CATIONS ON TISSUE WATER AND ELECTROLYTE DISTRIBUTION IN KIDNEY CORTEX SLICES LEACHED AT 0°

Slices leached 2.5 h at 0° in standard saline (see Experimental section) or in salines in which NaCl was equivalently replaced by 0.154 *M* solutions of other alkaline chlorides.

Exp. No.	*Cation (mEqu/l)*	*H_2O (ml/100 g DS)*	*Na^+*	*K^+*	*Cl^-*
			(mEqu/100 g DS)		
1	142.5 Na^+	406	53.6	16.2	45.5
	72.4 K^+	484	36.6	48.2	53.2
	144.8 K^+	510	14.0	76.1	54.5
	60.0 Rb^+	437	36.9	—	51.1
	120.0 Rb^+	491	13.9	—	56.6
	78.0 Li^+	426	39.4	18.1	42.1
	145.0 Li^+	433	9.0	12.9	42.8
2	142.5 Na^+	409	61.3	13.4	46.9
	149.0 K^+	499	5.1	76.5	66.0
	149.0 Rb^+	440	5.8	—	54.5
	149.0 Li^+	409	5.1	8.7	46.5



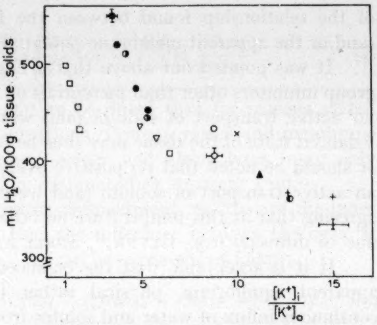


Fig. 4. Relationship of tissue water as a function of apparent Donnan ratio of potassium: for conditions of experiment and symbols see Fig. 3.

Fig. 3. Relationship of tissue water as a function of apparent Donnan ratio of chloride: tissue leached 2.5 h at 0° in standard saline (see EXPERIMENTAL) and modified salines with following additions: ○, control; ●, 0.2–0.6 *mM* $HgCl_2$; ◑, 0.7–1.4 *mM* Esidrone; □, 6–149 mEqu KCl replacing NaCl in the saline; ■, 67 and 134 mEqu RbCl replacing NaCl in the saline; ▽, 134 mEqu LiCl replacing NaCl; +, 1-6 m*M* $CaCl_2$; ▼, 6 *mM* $SrCl_2$; ▲, 6 *mM* $BaCl_2$; ◐, 6 *mM* $MnCl_2$; ×, 6 *mM* $MgCl_2$. Lines represent S.E. for both tissue water and apparent chloride ratio ($n = 10$) for following conditions: ├○┤, standard saline; ├●┤, 0.6 *mM* $HgCl_2$; ├+┤, 6 *mM* $CaCl_2$.

The relationship between tissue water and the apparent Donnan ratios of chloride and potassium

Reference has been made to the findings that whilst mercurial preparations increase the swelling of kidney cortex slices at 0° and decrease the apparent Donnan ratios of chloride and potassium, Ca^{2+} and some other alkaline earths have the opposite effect (KLEINZELLER AND CORT[10,11]). It was considered of interest to relate the apparent Donnan ratio of chloride and potassium to tissue water after leaching the slices in the presence of various agents. The results are shown in Figs. 3 and 4. It will be seen that, compared with controls leached in the standard saline, an increased apparent Donnan ratio of chloride, brought about by 1–6 *mM* Ca^{2+} (or other alkaline earths), corresponds to a decreased swelling of tissue; on the other hand, agents producing increased swelling of the slices, *i.e.* varying concentrations of K^+, Rb^+, 0.2–0.8 *mM* $HgCl_2$ and 0.7–1.4 m*M* Esidrone, also showed a decreased apparent Donnan ratio of chloride. A similar relationship holds for the apparent Donnan ratio of potassium and tissue water.

Since it is known that the Donnan ratio of freely diffusible ions is a determinant of the membrane potential, the results presented above suggest a relationship between the membrane potential of kidney cortex cells at 0° and the degree of cellular swelling.

DISCUSSION

Two aspects of the swelling of kidney cortex slices will be considered here, *i.e.* factors determining the apparent equilibrium of tissue components with the surrounding medium at 0° (the balanced state of the tissue) and the physico-chemical aspects of the relationship found between the Donnan ratios of chloride and potassium (and/or the apparent membrane potential), and the cell water.

It was pointed out above that a negative result with dinitrophenol and thiol-group inhibitors other than mercurials on tissue swelling at 0° suggest the view that no active transport of sodium (and water) takes place at this temperature. The balanced state of the tissue may thus be taken to represent an apparent equilibrium. It should be noted that no positive evidence is available for the opposite view, *i.e.* an active transport of sodium (and water) at 0°, most of the authors in the field agreeing that at this temperature no other transport processes take place but those due to diffusion (*e.g.*, GLYNN,[17], SNELL AND LEEMAN[18], CORT AND KLEINZELLER[19]).

If it is acceptable that the balanced state of the tissue at 0° represents an apparent equilibrium, physical rather than chemical factors counteracting the continuous influx of water and solutes from the leaching medium into the cells, due to Donnan forces, have to be considered. It is suggested, in accord with LEAF[6] that the distensibility (or elasticity) of the cellular membrane and intracellular structures (or possibly connective tissue surrounding the cell) might represent such a factor. As far as the author is aware there now exists some direct experimental evidence in favour of such a view: thus ERNST AND TIGYI[20] found that swelling of muscle in isotonic NaCl at 0° is significantly lowered by slight stretching, this of necessity changing the mechanical properties of the cellular membrane and intra-cellular structures (or connective tissue surrounding the muscle fibres).

There is now considerable evidence available that the cellular membrane of kidney cortex cells is freely permeable to K^+ (CORT AND KLEINZELLER[21]) and results presented above), Cl^- (WHITTAM[22], KLEINZELLER AND CORT[9]) and at 0° also to Na^+ (CORT AND KLEINZELLER[19]). Since these ions represent the bulk of those present in the leaching medium used in the experiments described above, and also the bulk of intracellular freely diffusible ions, it may be feasible to consider only their distribution. The following treatment, although necessarily a simplification, follows and extends arguments of LEAF[6] and BOYLE AND CONWAY[12].

A two-compartment system, separated by a semipermeable membrane, and at equilibrium, is assumed. Furthermore, the following simplifying assumptions are made: (a) none of the freely permeable ions are bound by components of the inner compartment (this assumption may not be fully valid for the cell); (b) activity coefficients equal 1.0; (c) ions in the inner compartment are evenly distributed (this assumption also may not be fully valid for the cell).

Let $[Na^+]_o$, $[K^+]_o$, $[Cl^-]_o$ denote molal concentrations of these ions in the outer compartment. Then

$$[Na^+]_o + [K^+]_o = [Cl^-]_o \tag{1}$$

For the inner compartment, sodium and potassium are the main cations, whilst Cl^- and non-diffusible anion A^{n-} represent the bulk of anions. Thus

$$[Na^+]_i + [K^+]_i = [Cl^-]_i + n[A^{n-}]_i \tag{2}$$

For a Donnan distribution the following relationship will hold:

$$\frac{[Na^+]_i}{[Na^+]_o} = \frac{[K^+]_i}{[K^+]_o} = \frac{[Cl^-]_o}{[Cl^-]_i} \tag{3}$$

and the membrane potential p will be:

$$\varphi = -\frac{RT}{F}\ln\frac{[\mathrm{Na}^+]_i}{[\mathrm{Na}^+]_o} = -\frac{RT}{F}\ln\frac{[\mathrm{K}^+]_i}{[\mathrm{K}^+]_o} = -\frac{RT}{F}\ln\frac{[\mathrm{Cl}^-]_o}{[\mathrm{Cl}^-]_i} \qquad (4)$$

When applying these terms to cells, it ought to be noted that for reasons stated above the values of intracellular ion concentrations, Donnan ratios and membrane potentials, calculated from analyses of tissue water and electrolytes, may only approximate actual values; they are therefore designated as apparent values.

Denoting the osmotic pressure in the outer compartment π_o, that of the inner compartment π_i, it is known (see LEAF[6]) that the difference between the osmotic pressures of both compartments, $\Delta\pi = \pi_i - \pi_o$, can be expressed as follows:

$$\Delta\pi = RT\,\{([\mathrm{Na}^+]_i + [\mathrm{K}^+]_i + [\mathrm{A}^{n-}]_i + [\mathrm{Cl}^-]_i) - ([\mathrm{Na}^+]_o \text{ [illegible] } [\mathrm{K}^+]_o + [\mathrm{Cl}^-]_o)\} \qquad (5)$$

We are now concerned with the question of the relationship of the Donnan ratio of chloride to $\Delta\pi$. By replacing in equation (5) the value of $([\mathrm{Na}^+)_o + [\mathrm{K}^+]_o)$ according to eq. (1), and the value of $([\mathrm{Na}^+]_i + [\mathrm{K}^+]_i)$ from eq. (2), and re-arranging, it will be seen that

$$\frac{\Delta\pi}{RT} = 2\,[\mathrm{Cl}^-]_i + (n+1)\,[\mathrm{A}^{n-}]_i - 2\,[\mathrm{Cl}^-]_o \qquad (6)$$

It thus follows that by maintaining experimental conditions, and consequently also $[\mathrm{Cl}^-]_o$ constant, the osmotic gradient between the inner and outer compartments should linearly increase with increasing $[\mathrm{Cl}^-]_i$. Since $\Delta\pi$ determines the influx of water into the inner compartment, an increased swelling of the inner compartment with increased $[\mathrm{Cl}^-{}_i]$ would follow, as demonstrated experimentally in Fig. 1 above, and by BOYLE AND CONWAY[12] for muscle.

In order to relate the ratio $\frac{[\mathrm{Cl}^-]_o}{[\mathrm{Cl}^-]_i}$ to $\Delta\pi$, a knowledge of the approximate value of $(n+1)[\mathrm{A}^{n-}]_i$ in eq. (6) is desirable. Assuming now that the number of charges per molecule of the non-diffusible anion is considerably greater than 1, we may write, using equation (2):

$$(n+1)\,[\mathrm{A}^{n-}]_i \doteq n[\mathrm{A}^{n-}]_i = [\mathrm{Na}^+]_i + [\mathrm{K}^+]_i - [\mathrm{Cl}^-]_i \qquad (7)$$

Thus by estimating the apparent values of $[\mathrm{Na}^+]_i$, $[\mathrm{K}^+]_i$ and $[\mathrm{Cl}^-]_i$, the approximate value of $(n+1)[\mathrm{A}^{n-}]_i$ can be obtained. In Table II the appropriate values are compiled as obtained by the author for rabbit kidney cortex, leached 2.5 h at 0° in the standard saline described above (KLEINZELLER AND CORT[11]), and as calculated from the data of WHITTAM (*cf.* LEAF[6]) for guinea-pig kidney cortex, leached for 2 h at 0° in a saline of similar composition. For the calculation of the data for rabbit kidney cortex the value of the inulin space was 0.20 ml per g tissue. The data of WHITTAM were calculated for an inulin space of 0.25 ml/g tissue (WHITTAM[22]); if the somewhat lower value of 0.20 ml/g, as found by CONWAY AND GEOGHEGAN[23] is taken, the agreement between both sets of values will be still closer.

It follows from Table II that there is satisfactory agreement of the apparent intracellular concentrations of the various components and also of the apparent Donnan ratios of chloride and potassium for both species. The relevant apparent value of $(n+1)[\mathrm{A}^{n-}]_i$ may be taken to approximate 90 mEqu/kg intracellular water.

TABLE II

APPARENT INTRACELLULAR ION CONCENTRATIONS IN KIDNEY CORTEX OF RABBIT AND GUINEA-PIG AFTER LEACHING AT 0°

The data for guinea-pig kidney cortex calculated from values of tissue water and electrolytes, found by WHITTAM, as quoted by LEAF[6]. Values for rabbit ± S.E. ($n = 10$).

Apparent ion conc. (mEqu/kg intracellular water)	*Kidney coertx*	
	Rabbit	*Guinea-pig*
$[Na^+]_i$	136.6 ± 2.2	132
$[K^+]_i$	45.2 ± 1.8	50.5
$[Cl^-]_i$	95.4 ± 2.4	82
$n[A^{n-}]_i = [Na^+]_i + [K^+]_i + [Cl^-]_i$	87.0 ± 3.4	100.5
$\frac{[K^+]_i}{[K^+]_o}$	8.86 ± 0.35	8.7
$\frac{[Cl^-]_o}{[Cl^-]_i}$	1.495 ± 0.037	1.64

On plotting the ratio $\frac{[Cl^-]_o}{[Cl^-]_i}$ against $\frac{\Delta\pi}{RT}$ for the above set of conditions, the hyperbolic curve shown in Fig. 5 is obtained. It will be seen that this curve corresponds to the trend of the relationship of tissue water as a function of the apparent $\frac{[Cl^-]_o}{[Cl^-]_i}$, as shown in Fig. 3.

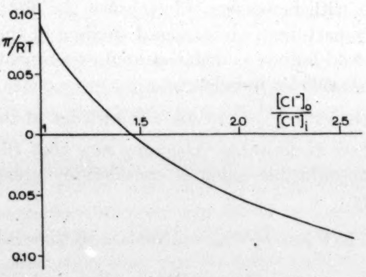


Fig. 5. Relationship of osmotic gradient as a function of the Donnan ratio of chloride: curve computed according to equation (6), for $(n + 1)[A^{n-}]_i = 90$ mEqu/kg intracellular water. For details see Text.

A similar relationship of $\Delta\pi$ as a function of $\frac{[K^+]_i}{[K^+]_o}$ might be derived, taking into account the Donnan distribution of freely diffusible ions (eq. 3). However, this relationship could not be verified experimentally for kidney cortex slices leached at 0°, the average apparent value of $\frac{[K^+]_i}{[K^+]_o}$ being 8.86 ± 0.35, that of $\frac{[Cl^-]_o}{[Cl^-]_i}$ 1.495 ± 0.037 and that of $\frac{[Na^+]_i}{[Na^+]_o}$ 1.1. It should be mentioned that it has been found in a number of tissues that apparent $\frac{[K^+]_i}{[K^+]_o} > \frac{[Cl^-]_o}{[Cl^-]_i}$, the directly measured membrane potentials corresponding to intermediate values (SHANES[24]). It may be assumed that the activity coefficient of intracellular K^+, as opposed to Cl^-, is lower than 1.0, possibly due to partial binding of potassium by intracellular components; such a view would explain the observation that K^+ cannot be completely removed from kidney cortex cells even by prolonged leaching in isotonic sodium chloride, while all tissue chloride can be readily removed by leaching the slices *e.g.* in isotonic sulphate (KLEINZELLER AND CORT[9]) or nitrate (unpublished results).

A simple Donnan system could not, however, explain the opposite effects of mercurials and alkaline earths. The increased swelling of kidney cortex slices in the presence of mercurial preparations cannot be explained by a Donnan distribution, taking into account their low effective concentrations (0.2–1.4 *mM*) and also their firm binding by tissue components. A Donnan distribution of Ca^{2+} would be expected to lead to an increased swelling of the tissue, since the relationship should hold:

$$\frac{[Cl^-]_o}{[Cl^-]_i} = \frac{\sqrt{[Ca^{2+}]_i}}{\sqrt{[Ca^{2+}]_o}}$$

However, the finding that both these groups of compounds have the opposite effect on the apparent Donnan ratios of chloride and potassium and on the membrane properties of kidney cortex cells, may point to the following tentative interpretation: if it is assumed that these compounds alter directly the ionic charge of the membrane by reacting with its components, then an inspection of eq. (4) will show that a corresponding redistribution of freely permeable ions would have to take place, with consequent changes of cellular hydration. Thus, *e.g.*, a direct increase of the membrane potential by Ca^{2+} would secondarily lead to an increase of the Donnan ratios of freely diffusible ions. Evidence for the localization of calcium at the cellular membrane of various cells has been summarized elsewhere (KLEINZELLER AND CORT[11], SHANES[24]) as well as evidence for calcium affecting the membrane potential of muscle and nerve tissue.

Finally, mention should be made of findings that also in some other cells opposite effects of mercurial preparations and calcium on cellular hydration were observed. Thus in brain cortex slices the tissue swelling in isotonic NaCl at 0° is increased by mercurial preparations (KLEINZELLER AND CORT[9]) and by potassium (KLEINZELLER AND RYBOVA[15]), while Ca^{2+} decreases swelling (RYBOVÁ[25]); also in yeast cells $HgCl_2$ in milimolar concentrations increases the cell volume at 0°, while Ca^{2+} decreases the volume (Dr A. KOTYK, personal communication). It would appear, therefore, that a common mechanism is involved in these changes.

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EFFECTS OF INSULIN ON MONOSACCHARIDE TRANSPORT AND INCORPORATION OF AMINO ACIDS INTO PROTEIN IN DIAPHRAGM DIFFERENTIATED WITH PHLORIZIN

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SUMMARY

The effects of phlorizin on monosaccharide transport, amino acid transport and amino acid incorporation into protein have been compared in isolated rat diaphragm. Conditions have been found under which phlorizin inhibits the uptake of glucose and membrane transport of D-xylose and D-galactose and the effect of insulin on these processes without affecting incorporation of [^{14}C]glycine into protein and the stimulation of this process by insulin. The results provide further evidence for the view that effects of insulin on amino acid incorporation are not dependent upon an effect of the hormone on carbohydrate metabolism.

INTRODUCTION

Insulin *in vitro* increases both uptake of glucose and incorporation of [^{14}C]amino acids into protein by isolated rat diaphragm muscle. There are points of evidence to

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suggest that the influence of insulin on amino acid incorporation is direct and not brought about through an effect on uptake of glucose[1–3]. In the present investigation further evidence in support of this view has been obtained by comparing effects of phlorizin on uptake of glucose and membrane transport of D-xylose and D-galactose and on incorporation of [^{14}C]glycine into protein and membrane transport of α-[^{14}C]aminoisobutyric acid and [^{14}C]glycine in isolated rat diaphragm.

METHODS

The methods and procedure for the preparation and incubation of intact and cut diaphragm preparations and for measurement of glucose uptake and xylose and galactose spaces are described elsewhere[4,5]. For measurement of xylose and galactose spaces intact diaphragms were incubated for 30 min at 37° in a bicarbonate buffered salt solution[6] pH 7.4 gassed with $O_2 + CO_2$ (95:5), containing either D-xylose or D-galactose at concentrations of 8.3 m*M* and 25 m*M* respectively. Uptake of glucose by the cut diaphragm preparation was measured by the disappearance of glucose from the medium (originally 2.5 mg/ml). Amino acid incorporation was measured by the method of MANCHESTER AND YOUNG[1], diaphragm being incubated for 2 h in the presence of [1-^{14}C]glycine (1 m*M* and specific activity of about 400 µC/mmole). Amino acid accumulation, *i.e.* the ratio of the concentration of the free labelled amino acid in the tissue to that in the medium, was measured as described by MANCHESTER AND YOUNG[7]. Phlorizin (British Drug Houses, Ltd.) was recrystallized twice from hot water before use, and dissolved directly in the medium. A five-times recrystallized sample of insulin (Boots Pure Drug Co., Ltd.) was dissolved in *N*/300 HCl to yield a stock solution containing 20 units/ml which was diluted to 0.1 unit/ml with buffer shortly before each experiment.

RESULTS

The effects of phlorizin on uptake of glucose, incorporation of [^{14}C]glycine into diaphragm protein and intracellular accumulation of xylose and galactose are shown in Tables I and II. The intracellular accumulations of D-xylose and D-galactose by the

TABLE I

EFFECT OF PHLORIZIN ON THE ACCUMULATION OF D-XYLOSE AND D-GALACTOSE BY THE INTACT DIAPHRAGM PREPARATION

Each figure is the mean ± S.E. of the mean of six observations. The value of P for a difference which is significant is indicated. Insulin was present at a concentration of 0.1 unit/ml in each instance.

Sugar added to the medium	*Phlorizin (3 mM)*	*Intracellular xylose or galactose space (ml/100 g of wet muscle)*	
D-xylose	—	28 ± 2.4	
	+	15 ± 1.8	
	Difference —	13 ± 3.0	($P < 0.01$)
D-galactose	—	28 ± 2.6	
	+	18 ± 2.0	
	Difference —	10 ± 3.3	($P < 0.01$)

TABLE II

EFFECT OF PHLORIZIN ON THE UPTAKE OF GLUCOSE AND INCORPORATION OF [^{14}C]GLYCINE INTO PROTEIN BY ISOLATED RAT DIAPHRAGM, BOTH IN THE PRESENCE AND ABSENCE OF INSULIN

Each figure is the mean ± S.E. of the mean of six observations. The value of P for a difference which is significant is indicated. The effect of insulin is significant in each instance ($P < 0.001$).

Addiiton to medium	*Glucose uptake (mg/g wet wt/h)*				*Radioactivity in diaphragm protein (counts/min/disc)*			
	No insulin added		*Insulin added (0.1 unit/ml)*		*No insulin added*		*Insulin added (0.1 unit/ml)*	
Intact diaphragm								
No addition							505 ± 12	
Phlorizin (3 m*M*)							493 ± 19	
Cut diaphragm								
No addition	2.75 ± 0.10	$P < 0.001$	6.30 ± 0.25	$P < 0.001$	383 ± 12	$P < 0.02$	582 ± 16	$P < 0.01$
Phlorizin (3 m*M*)	2.06 ± 0.09		3.60 ± 0.09		338 ± 9		484 ± 17	
No addition	1.78 ± 0.06	$P < 0.001$	4.45 ± 0.17	$P < 0.001$	404 ± 26		622 ± 30	
Phlorizin (1 m*M*)	1.14 ± 0.08		3.21 ± 0.11		424 ± 30		646 ± 19	

intact diaphragm (measured in the presence of insulin) were reduced by 50 % and 30 % respectively in the presence of phlorizin (3 m*M*) (Table I). The incorporation of [^{14}C]glycine into protein in the intact diaphragm preparation (measured in the presence of insulin) was unaffected by phlorizin (3 m*M*) (Table II). Uptake of glucose by the cut diaphragm preparation was inhibited by phlorizin (3 m*M*) 25 % in the absence and 40 % in the presence of insulin. The insulin effect on glucose uptake was thus inhibited 60 % by 3 m*M* phlorizin (Table II). Incorporation of [^{14}C]glycine by the cut diaphragm preparation was inhibited 12 % by 3 m*M* phlorizin in the absence and 17 % in the presence of insulin. The insulin effect on glycine incorporation was thus inhibited 25 % (Table II). Phlorizin at 1 m*M* concentration inhibited uptake of glucose by the cut diaphragm preparation 30 % in the absence and 25 % in the presence of insulin (22 % inhibition of the insulin effect) (Table II). At this concentration phlorizin had no effect on glycine incorporation in the cut diaphragm preparation in the presence or absence of insulin. These results show that phlorizin can inhibit uptake of glucose and membrane transport of D-xylose and D-galactose in isolated diaphragm, and also effects of insulin thereon, under conditions in which it has no demonstrable effect on incorporation of [^{14}C]glycine into protein.

The incorporation of labelled amino acids into diaphragm protein involves both transport of the amino acid across the cell membrane and its participation in peptide bond formation. The rate of entry of labelled amino acids from the medium into diaphragm appears to be a rapid process[7] and it appears not to be the rate-limiting step in the overall process of transfer of labelled amino acids from the medium into diaphragm protein[7-9]. Because it was therefore possible that phlorizin might inhibit amino acid transport in diaphragm without inhibiting the overall process of amino acid incorporation into protein, the effects of phlorizin on the accumulation of glycine and α-aminoisobutyric acid in diaphragm were also investigated.

Phlorizin (3 m*M*) was found to depress the accumulation both of [^{14}C]glycine and of α-[^{14}C]aminoisobutyric acid by the cut diaphragm preparation (Table III),

TABLE III

EFFECT OF PHLORIZIN (3 mM) ON THE ACCUMULATION OF [^{14}C]AMINO ACIDS BY INTACT AND CUT RAT DIAPHRAGM PREPARATIONS

Each figure is the mean ± S.E. of the mean of six observations.

Amino acid added to the medium	*Ratio: Concentration of free labelled amino acid in tissue water / Concentration of free labelled amino acid in incubation medium*		*Significance of difference (P)*
	No phlorizin added	*Phlorizin added*	
Intact diaphragm preparation			
α-[1-^{14}C]aminoisobutyric acid	0.77 ± 0.021	0.76 ± 0.026	N.S.*
[1-^{14}C]glycine	1.61 ± 0.11	1.56 ± 0.063	N.S.*
Cut diaphragm preparation			
α-[1-^{14}C]aminoisobutyric acid	2.35 ± 0.065	1.92 ± 0.068	= 0.001
[1-^{14}C]glycine	3.83 ± 0.099	3.34 ± 0.075	< 0.001

* Not significant ($P > 0.1$).

but it had no significant effect on the accumulation of these amino acids by the intact preparation (Table III). Thus under conditions in which incorporation of glycine into protein is unaffected by phlorizin, there appears equally to be no effect of phlorizin on amino acid transport; on the other hand, under comparable conditions phlorizin markedly depresses the transport of glucose, xylose and galactose.

DISCUSSION

The present results show that, whereas phlorizin inhibits monosaccharide transport in both the cut and the intact diaphragm preparation, it only inhibits amino acid transport and incorporation into diaphragm protein in the cut preparation. An important difference between the cut and intact diaphragm is that substances which are confined to extracellular water in the latter preparation appear to penetrate muscle cells in the former, possibly through the cut ends of muscle fibres[4,10]. In the intact diaphragm phlorizin appears to be confined to extracellular water[5]. This would suggest that its effects on monosaccharide transport are exerted at the surface of the muscle cell and, conversely, that it has no effect on amino acid transport or incorporation at this locus. If, as seems probable, phlorizin penetrates the cut diaphragm preparation, then its effects on amino acid transport and incorporation in this preparation may result from an intracellular action. KELLER AND LOTSPEICH[11] have claimed that phlorizin inhibits oxidative phosphorylation in isolated mitochondria. Because amino acid transport and incorporation into protein are dependent upon energy-rich phosphate, effects of phlorizin on these metabolic processes could be secondary to an effect of phlorizin on oxidative phosphorylation in mitochondria in the cut diaphragm preparation.

The results presented here provide a clear demonstration of the separate nature of the effects of insulin on monosaccharide transport and on amino acid incorporation. They support the view previously advocated[1-3] that stimulation by insulin of amino acid incorporation into diaphragm is not dependent upon an effect of the hormone on glucose uptake. The evidence for this view may be briefly summarized:

1. Insulin *in vitro* stimulates incorporation of labelled amino acids into the protein

of isolated diaphragm from the normal[1,2], hypophysectomized[3], adrenalectomized[3] or alloxan-diabetic[12] rat. Such an effect is equally visible whether glucose is present in the medium or not. Addition of glucose or pyruvate does not produce an observable augmentation of incorporation, nor does the presence of these substances modify the size of the insulin effect[1,2,13].

2. Hypophysectomy augments, and treatment of the hypophysectomized or intact rat with pituitary growth hormone diminishes, the magnitude of the stimulation of glucose uptake by isolated diaphragm produced by insulin, whereas these same operations either have no effect or produce slightly the reverse effect on the sensitivity of isolated diaphragm to an effect of insulin on amino acid incorporation —that is, hypophysectomy may decrease, and treatment with growth hormone increase, the size of the effect of insulin on this process[3].

At first sight acceptance of these views implies that insulin has two separate and distinct effects on muscle metabolism—on protein synthesis and on monosaccharide transport respectively. However, if, as has been suggested by RANDLE AND SMITH[4,14], insulin accelerates transport of glucose in diaphragm by restricting the access of energy-rich phosphate to a process concerned with the regulation of glucose transport, it may well be that this same action of insulin makes energy-rich phosphate more freely available to processes involved in protein synthesis. But at the present time such an hypothesis is purely conjectural.

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AMINO ACID STARVATION IN AN *ESCHERICHIA COLI* AUXOTROPH

II. ACID-SOLUBLE PURINE AND PYRIMIDINE DERIVATIVES IN NORMAL AND STARVED CELLS

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SUMMARY

1. The acid-soluble purine and pyrimidine compounds of a leucine auxotroph of *E. coli* K_{12} have been fractionated on Dowex-2 formate, followed by paper chromatography and electrophoresis.

2. The acid-soluble fraction contains large amounts of free hypoxanthine and uracil, as well as nucleotide co-enzymes and some nucleoside di- and tri-phosphates, but has only very small amounts of nucleoside monophosphates. Derivatives of adenine and uracil predominate.

3. Leucine starvation increases the concentrations of most but not all of the bases and nucleotides. No new nucleotide compounds have been found in starved cells.

INTRODUCTION

POTTER *et al.*[1,2] found acid-soluble ribonucleoside mono-, di-, and tri-phosphates in rat liver cells. Their methods were applied to yeast by SCHMITZ[3] and to *Escherichia coli* by O'DONNELL *et al.*[4]. The same compounds were found in all these organisms, though apparently in different amounts.

Acid-soluble purine and pyrimidine derivatives accumulate when an amino acid auxotroph of *E. coli* has exhausted its amino acid supply, although there is no net synthesis of protein or of RNA[5,6]. The nature of the excess material was of interest because it might possibly contain RNA components specifically related to the missing amino acid. Accordingly we have analysed the acid-soluble pool both in exponentially-growing cells and in amino acid starvation. We find that the changes involve many of the normal ingredients of the pool and are probably not related to the mechanism for incorporating a particular amino acid. No new compounds were found in starved cells. Probably the whole effect is best regarded as the buildup and diversion of RNA precursors due to the cessation of RNA synthesis.

Abbreviations: RNA, ribonucleic acid; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; UMP, uridine monophosphate; UDP, uridine diphosphate; UTP, uridine triphosphate; GMP, guanosine monophosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; CMP, cytidine monophosphate; CDP, cytidine diphosphate; CTP, cytidine triphosphate.

METHODS

A leucine-requiring mutant of *E. coli* was used; it was "starved" by exhaustion of leucine as previously described[5]. 15 l of mineral-glucose medium was inoculated with 200 ml of a 16-h culture. Control cultures containing 20 μg/ml of L-leucine were harvested after 3.5 h at a bacterial density of about 0.1 mg/ml. "Starved cell" cultures, containing 5 μg/ml leucine, grew at the same rate as the controls until leucine was exhausted, at 3.5 h. Turbidity then increased only 10 % until the time of harvest, 90 min later.

All subsequent procedures were the same for normal and starved cells. The cells were rapidly chilled, harvested on the Servall SZENT-GYÖRGYI-BLUM continuous centrifuge, washed with 0.15 % NaCl and suspended in water. Trichloroacetic acid was added to a concentration of 5 %. After 1 h at 5°, the precipitate was centrifuged off, and the supernatant (150 ml for 1.0 to 1.5 g cells) was freed of trichloroacetic acid with ether and neutralized to pH 6 to 7.

Separation on anion exchange resin

The first rough separation was carried out rapidly on a column, 2 × 8 cm, of Dowex-2-X10 formate (100–200 mesh), using chilled eluting solutions. 15-ml fractions were collected at 5 ml/min, and their O.D. determined at 260 mμ. They were chilled, pooled into 5 peaks, and dried rapidly in the flash evaporator. The whole column and drying operation took about 4 h. Peak 1 is the material washed through the column with distilled water. The column was then eluted with 3 concentrations of formic acid: 0.05 *N* (peak 2), 1.0 *N* (peak 3), and 5.0 *N* (peak 4), and with 0.5 *N* HCl, (peak 5)*. The final eluate, in 2.5 *N* HCl, did not have a nucleotide spectrum; it was discarded. Recovery in peaks 1–5 averaged 90 % of the original u.v. absorption in the control runs and 86 % for the starved cells.

Paper chromatography and electrophoresis

The solvents used for chromatography were: (a) isopropanol–concentrated formic acid–water (100:9:34), (b) saturated ammonium carbonate solution–water–isopropanol (3:1:6), (c) isobutyric acid–conc. ammonia–water (66:1:33), and (d) 60 % ammonium sulfate with 0.1 *M* Tris and 2 % isopropanol, pH 7. Material from the column was chromatographed in 2 dimensions, usually in solvents a and b. Unresolved portions were dried and subjected to rechromatography in solvents c or d, and sometimes to paper electrophoresis in 0.1 *N* formic acid.

Spots were located by quenching of u.v. light (Mineralight, Model SL2537), and eluted with dilute HCl. The spectra were read in the Beckman recording spectrophotometer, model DK-2. Recoveries were calculated from the O.D. at 260 mμ. The amounts of each compound reported here are the recoveries from the first 2-dimensional chromatogram of each peak. In cases of incomplete separation at this stage, the eluted u.v. absorption indicated the total amount of the mixture and further steps established its composition. Due to loss on the column, poor (about 65 %)

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* HCl was used instead of formic–formate mixtures because the latter are too salty for satisfactory rechromatography on paper. Exposure to 0.5 *N* HCl and drying from the HCl solution caused very little breakdown of samples of commercial ATP. (But about 30 % appeared as ADP after several paper chromatography steps.)

recovery from papers, and removal of non-nucleotide material, the amounts listed total only about 0.5 of the original u.v. absorption of the acid-soluble fraction.

Phosphate was determined by a modification[7] of the method of FISKE AND SUBBAROW. Ninhydrin sprays and a spray for amino sugars[8] were done on various compounds in peaks 4 and 5. DPN and TPN were identified by the peak at 327 mμ on addition of cyanide[9].

We have not distinguished ribose from deoxyribose compounds. Previous work[5] showed that acid-soluble deoxyribose amounts to about 10 % of the ribose. Part of this is accounted for here by thymidine derivatives; the rest may be included in various nucleotide fractions.

RESULTS

In preliminary experiments, acid-soluble fractions from leucine, proline, and tyrosine auxotrophs of *E. coli* K_{12} were compared by examining the u.v. absorption in 11 peaks obtained by gradient elution with HCl from a Dowex-2 chloride column. The mutants, when supplied with amino acids, gave patterns similar to the wild type. When starved of their required amino acids, all 3 mutants accumulated nucleotide material, and the increases in each peak were similar for the different strains. The rest of this report deals with the leucine auxotroph only.

Separation on Dowex-2 formate

Typical elution patterns of the acid-soluble fractions of normal and starved cells are shown in Fig. 1. Peak 1, the material not retained by the column, is 90 % larger in starved than in normal cells, and constitutes about 20 % of the recovery in both cases. Peak 2, about 0.25 of the total, is more than doubled in starvation.

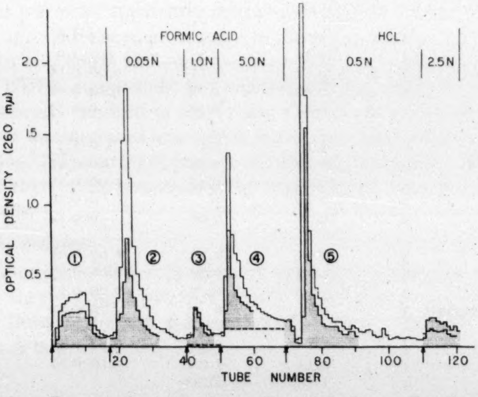

Fig. 1. Elution from Dowex 2-formate column. Two typical runs. Fraction volume 15 ml. Flow rate 5 ml/min. The 5 peaks were eluted with distilled water, formic acid and HCl as shown at the top of the figure. The u.v. absorption of the eluting solutions was negligible except in peaks 3 and 4 where it is indicated by a broken line. O.D. was read against distilled water. The stippled histogram represents normal cells; the open histogram represents starved cells. Fewer tubes were needed in each peak for normal cells; the peaks have been superimposed by breaking the curve for normal cells in several places.

Peak 3 is very small and does not change significantly. Peak 4 is similar in size to peaks 1 and 2, and it increases about 50 % in starvation. Peak 5 is the largest fraction, about a third of the total, and shows a 65 % increase in starved cells.

Compounds isolated from paper chromatograms

Table I shows the compounds identified in 3–5 runs of each type of cell.

Peak 1: In peak 1, hypoxanthine and uracil each made up about 45 % of the recovery. The remainder was guanine, adenine and adenosine. Both the major components were approximately doubled in amount by starvation. The apparent increases in guanine and adenosine do not exceed the variability of the method.

Peak 2: The bulk of peak 2 was DPN. Cyanide tests on the whole peak before drying showed enough DPN to account for almost all the adenosine content of the peak. The observed AMP may in part be derived from DPN.

Peak 2 also contained low levels of CMP, not increased by starvation. Free xanthine was present in small amounts; it may be increased in starvation. A compound with a xanthosine spectrum, presumably the nucleoside itself, was found in 2 of the 3 runs with normal cells and in none of the starved cells.

Peak 3 was not affected by starvation. It contained adenosine compounds,

TABLE I

RESULTS OF THE CHROMATOGRAPHIC SEPARATION OF COMPOUNDS IN EACH OF THE COLUMN FRACTIONS

The data are derived from 3 to 5 pairs of experiments.

		µmoles/g of cells			
Compound	*Peak*	*Average*		*Range*	
		Normal	*Starved*	*Normal*	*Starved*
Hypoxanthine	1	1.6	2.5	1.5–1.8	2.2–3.3
Uracil	1	1.3	2.4	0.7–1.9	2.1–2.6
Adenine	1	0.12	0.17	0.06–0.15	0.05–0.32
Adenosine	1	0.06	0.24	0.04–0.08	0.21–0.30
Guanine	1	0.04	0.22	0–0.13	0–0.36
DPN	2	1.3	3.1	1.0–1.5	2.2–3.7
AMP	2	0.20	0.35	0–0.49	0.09–0.77
Xanthine	2	0.20	0.45	0.06–0.29	0.27–0.72
Xanthosine	2	0.13	0	0–0.26	—
CMP	2	0.06	0.06	0–0.14	0–0.18
ADP	3	0.13	0.30	0.07–0.21	0.22–0.37
AMP	3	0.20	0.30	0.08–0.25	0.25–0.35
DPN	3	0.12	0.07	0.09–0.15	0.04–0.10
Xanthosine monophosphate	3	0.03	0	0.02–0.04	—
ADP + TPN	4	1.1	1.3	0.32–1.4	0.55–2.9
UDPX	4	0.53	1.9	0.19–0.77	1.8–2.6
UMP	4	Trace	Trace		
UDP + UTP	5	0.92	2.1	0.67–1.0	1.0–2.7
ATP	5	1.6	2.8	0.86–2.1	2.3–3.3
GDP + GTP	5	0.53	0.58	0.30–0.68	0.05–0.90
CTP	5	0.47	2.0	0.39–0.55	0.56–3.0
Thymidine triphosphate	5	Trace	Trace		

isolated as AMP, ADP, and DPN or TPN. In normal cells there were also traces of another xanthosine compound.

Peak 4 contained several compounds of adenosine and uridine. The former included ADP and TPN. Cyanide tests showed no significant increase in TPN in starved cells, nor was there any change in the total adenosine content of peak 4.

Almost all the uridine compounds isolated from peak 4 had 2 phosphates per mole of base. A trace of UMP was sometimes isolated. The presence of UDP-amino sugar compounds was indicated by spray reactions. There were ninhydrin-positive spots with R_F values unlike glucosamine or N-acetylglucosamine. They reacted like the latter with the amino sugar spray. They resemble an acetyl-amino sugar-amino acid complex such as that found in PARK's[10] nucleotide 3. All UDP compounds in peak 4 are considered together as "UDPX". They were increased about four-fold during starvation. UDP itself was eluted in peak 5 (see below).

Peak 4 contained a number of strongly fluorescent compounds, some with spectra similar to guanosine. However, rechromatography failed to separate out any pure GMP. Spectra resembling thymidine (presumably as thymidylic acid) and orotic acid were occasionally found.

Peak 5: In peak 5 were found compounds of all four RNA bases, including nucleoside tri-, di- and monophosphates, and sometimes free adenine and guanine. Breakdown during chromatography obscured the relative amounts of the di- and triphosphates. Control runs with authentic compounds established that ADP comes out in peak 4 (CDP would be expected to come out before ADP), but UDP, GDP and all 4 nucleoside triphosphates appear in peak 5. We have therefore assumed that all peak 5 adenosine and cytidine compounds represent ATP and CTP respectively. Uridine and guanosine compounds probably include both the di- and triphosphates. We found UDP, UTP, and GDP, and some guanosine compounds with 2.5 phosphates per mole, apparently mixtures of GDP and GTP. It appears that the pools of UTP and GTP are small relative to the diphosphates, but better separations are needed. An occasional ninhydrin-positive UDP spot indicated that peak 5 may contain some UDPX compounds, but much less than peak 4.

ATP was nearly doubled in starvation. CTP is not a prominent component of normal cells but was increased four-fold in starvation. Starvation produced a doubling of the UDP-plus-UTP content of peak 5, but did not change the GDP-plus-GTP at all.

Traces of thymidine, presumably the triphosphate, were found in both normal and starved cells.

Excretion to the medium

During starvation, the cells excrete more u.v.-absorbing material into the medium than they do during normal growth. Most of the excreted material can be eluted from a Dowex-2 formate column in peaks 1 and 4. We have not yet examined either peak in detail.

DISCUSSION

Normal, exponentially growing cells

The base ratios for the entire normal nucleotide pool in *E. coli* K_{12} are: adenine 1.0, uracil 0.6, hypoxanthine 0.4, and guanine, cytosine and xanthine each about 0.1.

The free bases of all the above compounds except cytosine were found in normal cells. Hypoxanthine and uracil were quite plentiful. We do not believe that they are artifacts; although hypoxanthine may be the result of inosinic acid breakdown, uracil is very unlikely to arise accidentally. These two bases have also been found in *E. coli* by Škoda and Šorm[11] and O'Donnell *et al.*[4]. Both can be rapidly incorporated into RNA when they are supplied in the medium, though they are not intermediates in the *de novo* synthesis of RNA. The situation may be analogous to that described by Cowie and Bolton[12] in yeast, where it appears that the nucleotide pool is limited in size but that a variable pool of free bases also exists, and this may be very large when exogenous purines are supplied. Perhaps free bases can be stored, and converted into nucleotides as needed. Their level may serve to regulate the size of the nucleotide pool by feedback control of *de novo* synthesis[13,14].

Nucleosides and, surprisingly, nucleoside monophosphates were absent or very scanty. Diphosphates of adenosine, guanosine, and uridine were all major components of the nucleotide pool, but no diphosphates of cytidine or thymidine were found. Nucleoside triphosphates do not appear to be plentiful in normal cells, with the exception of ATP. Nucleotide co-enzymes, especially DPN and various UDPX compounds, formed an unexpectedly large proportion of the total pool.

Leucine-starved cells

During starvation the levels of hypoxanthine and uracil rose even higher than in normal cells. Nucleosides and their monophosphates were not accumulated, nor were any of the diphosphates except UDP. Among the nucleoside triphosphates, ATP and CTP were increased by starvation and UTP was probably also increased, but GTP was not. DPN and UDPX were increased, but there was no change in TPN levels.

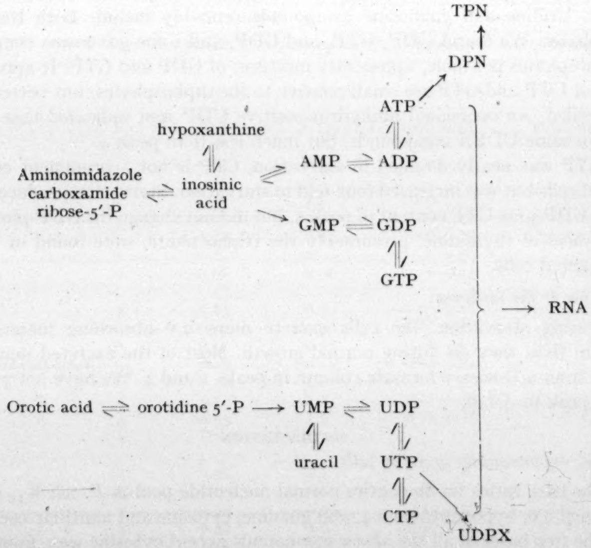

Fig. 2. Scheme of interrelations of acid-soluble purine and pyrimidine compounds.

One object of this work was to determine whether the accumulated material would give any information about the coding for leucine on the presumed RNA template. It was conceivable that these compounds represented partial breakdown of RNA, specifically at the sites of leucine incorporation. The following considerations indicate that the major changes observed are not specific for leucine deprivation: (a) column elution patterns were similar for cells deprived of leucine, tyrosine or proline; (b) many different compounds accumulated, and (c) these included substances which are neither precursors nor products of RNA, but (d) there were no unique compounds such as oligonucleotides or amino acid-nucleotide complexes. The accumulation of purine and pyrimidine derivatives during amino acid starvation probably represents some rather general secondary effects of the blockade of RNA synthesis. The mechanism by which amino acid starvation inhibits RNA synthesis is unknown. The present results show that the direct precursors of RNA, whether these are the nucleoside di- or tri-phosphates, are in good supply, so the cause of the block must be sought outside the acid-soluble fraction.

Fig. 2 is a rough scheme of RNA and co-enzyme synthesis. When RNA synthesis is blocked, there should be a buildup of nucleoside di- or tri-phosphates. Since the machinery for oxidative phosphorylation should be unimpaired, it is not surprising that the triphosphates and their derivatives, DPN and UDPX, should accumulate. (TPN and GTP should also accumulate; the scheme does not explain their failure to do so.) The observed increases in hypoxanthine and uracil indicate that inosinic acid and UMP continue to be formed but are sidetracked, again suggesting that the bases are storage forms of nucleotide material.

ACKNOWLEDGEMENTS

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PROTEIN AND AMINO ACID TURNOVER DURING DIFFERENTIATION IN THE SLIME MOLD

I. UTILIZATION OF ENDOGENOUS AMINO ACIDS AND PROTEINS

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SUMMARY

During starvation and multicellular differentiation, the slime mold sequentially utilizes endogenous amino acids, ethanol soluble and ethanol insoluble protein. The amino acid pool drops to approximately 30 % of its initial value, the ethanol soluble protein to 60 % and the ethanol insoluble protein to about 80 % of its initial value during the course of differentiation.

INTRODUCTION

The slime mold, *Dictyostelium discoideum*, multiplies as individual myxamoebae by engulfing bacteria on a complex medium[1,2]. When the myxamoebae have consumed the available bacteria, the cells can be washed and spread on purified agar in order to observe the subsequent stages of their life cycle in the absence of any nutrients. In response to a diffusible hormone, the cells aggregate at various points on the plate to form multicellular pseudoplasmodia[2,3]. Further differentiation of this new "organism" proceeds during starvation of its cells. They begin to respire more actively, and to utilize, with increasing intensity, endogenous proteins as a source of energy and building blocks for the synthesis of carbohydrates[4,5]. Histochemical studies[6,7] as well as *in vitro* analyses[8,9] demonstrate that a number of enzymes change in activity during development. The exact extent of these changes is difficult to ascertain, since enzyme activities in cell-free extracts display different degrees of stability, depending on the stage of differentiation from which they are prepared[10].

In order to describe and analyze further some of the biochemical events accompanying morphological differentiation, studies have been carried out on the manner in which endogenous amino acids and proteins are utilized.

MATERIALS AND METHODS

L-[^{35}S]*methionine* was obtained from Abbott Laboratories. Chromatographic and microbiological analyses demonstrated that this material is 100 % L-methionine.

Growth of D. discoideum

The procedures used for growing the slime mold and for following various biochemical changes of the multicellular unit in the absence of growth have been previously described[11]. In the present studies, the myxamoebae were always grown on *E. coli* strain ML304d which has an absolute requirement for L-methionine. The *E. coli* mutant was grown in a minimal medium supplemented with an excess of L-[^{35}S] methionine (0.1 µmoles/ml) and then mixed with spore heads of *D. discoideum* on complex media in the usual manner. The myxamoebae which subsequently grew were thus labeled by ingestion of the bacteria as well as by the excess labeled methionine present in the complex media. All the experiments to be described were performed after the myxamoebae were washed and incubated on purified agar with no supplements.

Protein and amino acids

These were measured by the biuret reagent[12] and by quantitative ninhydrin determinations[13] respectively.

Methionine assay

E. coli Strain ML304d was used to determine methionine quantitatively. A total volume of 5.0 ml minimal medium supplemented with various amounts of methionine was shaken overnight at 37° in a 50-ml Erlenmeyer flask and the density of the culture read in the Klett-Summerson colorimeter with a blue filter (420 mµ). Under these conditions the growth response to L-methionine is linear at concentrations from 0.005 µmoles to 0.06 µmoles.

Fractionation of cells

The cells were fractionated into the following general classes of compounds: amino acid pool and other small molecules, nucleic acid and carbohydrates, ethanol soluble protein and residual, ethanol insoluble protein. The method of SCHNEIDER, as modified by ROBERTS *et al.*, was used [14,15]. It was found that each class of compounds was essentially homogeneous and uncontaminated by other fractions.

EXPERIMENTAL RESULTS

[^{35}S]methionine was chosen as a specific label of amino acids and proteins since the extensive carbohydrate synthesis from the carbon skeleton of amino acids occurring in the later stages of differentiation obscured results with [^{14}C]amino acids. Preliminary information was obtained for more than one class of protein by extraction of the proteins precipitated by TCA with ethanol. The fate of ethanol soluble protein was investigated because of its preferential utilization in *E. coli* under starvation conditions[16].

Overall loss of ^{35}S during development

During differentiation of [^{35}S]methionine-labeled cells on washed agar, there is a gradual loss of total counts with time per aliquot of harvested slime mold. In order to account for this loss in ^{35}S activity, petri dishes with labeled amoebae were inverted over water, to trap any volatile material. After fruiting had occurred, the counts in the slime mold, the agar and the water were compared to the counts of an original control culture which had been harvested immediately after preparation. The fruits had retained 63% of the original counts, the agar 36% and the water trap 2%. The ^{35}S material in the agar has not yet been identified.

Distribution of [^{35}S]methionine in amoebae

Table I gives the percentage distribution of total counts in the various chemical fractions obtained from amoebae labeled with [^{35}S]methionine (see METHODS). The last experiment was done with an aggregateless mutant obtained from Dr. M. SUSSMAN[17]. Qualitatively similar count distributions have been observed for *E. coli* cells labeled with ^{35}S during exponential growth[15]. In *E. coli*, however, the amino acid pool contains only 2 % of the total counts since methionine is present mainly in the proteins of these cells. The size of the amino acid pool in the slime mold is particularly dependent upon how quickly the cells are harvested after being plated on washed agar, since of the four classes of compounds studied, "free" amino acids begin to be used very early during development. In these experiments, the amoebae were harvested for analysis between one and 3 h after plating.

TABLE I

% DISTRIBUTION OF [^{35}S]METHIONINE IN AMOEBAE

Expt.	*Strain*	*Amino acid pool*	*Et. sol prot.*	*Et. insol. prot.*	*nucleic acid**	*% recovered*
I	Wild	9.0	12.8	67.8	2.7	89
II	Wild	17.0	10.0	56.0	3.3	86
III	Wild	12.3	15.5	71.0	2.1	100
IV	Agg 204	8.7	18.0	80.0	2.6	100

* The radioactivity in this fraction may be attributable to thiomethyl adenosine[11,17].

Specific radioactivity determinations

In order to interpret experiments to be described, it was necessary to determine whether or not methionine was present as a constant fraction of the amino acid pool and of protein at all stages of development. ^{35}S-labeled amoebae were plated on purified agar. At the amoeba, slug and fruit stages a number of plates were harvested, and the cells frozen and homogenized to obtain a cell-free supernatant as well as a precipitate fraction containing the cell debris. Protein which could be obtained from the cell debris fractions gave values similar to those found for the companion supernatant fraction. Table II gives specific radioactivity data for the amino acid pool, the ethanol soluble and ethanol insoluble protein. The data demonstrate that methionine is present as a reasonably constant fraction of the amino acids in the pool and in the two classes of proteins at the slug compared to the amoeba stage, but that significant differences may exist when comparing the slug and fruit stages with respect to the amino acid pool and the ethanol insoluble protein. Chromatographic analysis has

TABLE II

SPECIFIC RADIOACTIVITIES OF PROTEINS AND AMINO ACID POOL

Stage	*Counts/min/mg protein*		*Counts/min/µmole amino acid*
	Et. insol.	*Et. sol.*	*Amino acid pool*
Amoebae	3,630	1,540	955
Slug	3,480	1,270	909
Fruit	4,700	1,230	630

shown that about 80–90 % of the radioactivity in the amino acid pool is methionine, and in the hydrolyzed protein is methionine or its chemical breakdown products. KRIVANEK AND KRIVANEK[18] have shown by paper chromatography that the free amino acid composition (including methionine) of the slime mold is essentially similar at the various stages of development.

In vivo *utilization of amino acids and proteins*

Experiments were carried out to obtain information concerning the relative behavior of the amino acid pool and the two types of protein during starvation and differentiation of the slime mold on washed agar. A typical experiment is shown in Fig. 1. The values are expressed as percent of the initial total radioactivity found for each class of compounds. The stage of development is indicated by the figures on the abscissa. By thus following the radioactivity in the various fractions obtained from cells harvested at progressive stages of development, it appears that the "free" amino acids are utilized first, then the ethanol soluble and finally the ethanol insoluble protein. Considering both classes of protein, these data indicate that the most active proteolysis occurs between the early slug and culmination stages.

The data of Table II indicate that corrections should perhaps be applied to the values for the fruit with respect to the ethanol insoluble protein and the amino acid pool. For the early stages of development, however, the general sequence of utilization of the three classes of nitrogenous compounds is clear.

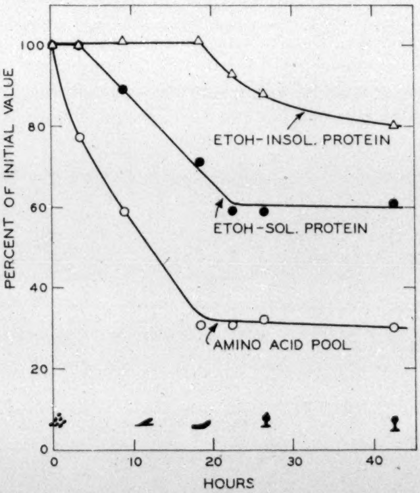

Fig. 1. Utilization of endogenous amino acids and protein during development. The cells were labeled with radioactive methionine and allowed to differentiate in petri dishes on washed agar. The figures on the abscissa indicate the following sequential stages: amoebae, early slug, slug, culmination and fruit. At various times during development, 3 plates (all prepared from the same number of amoebae originally) were harvested and fractionated into the amino acid pool, ethanol soluble and ethanol insoluble protein. Each point is expressed as the percent of the initial radioactivity for that fraction.

DISCUSSION

It is not surprising to find that starvation of the slime molds brings about an early utilization of the amino acid pool. In yeast[19] and *E. coli*[20] the pool amino acids are incorporated into protein during nitrogen starvation. It will be apparent from the accompanying paper that this in part is the fate of "free" amino acids in *D. discoideum*. The preferential utilization of ethanol soluble protein also has its analogy in the metabolism of sulfur-starved *E. coli* cells[16].

In the differentiating slime mold, the utilization of amino acids and protein takes on an added significance, in the sense that it not only accompanies starvation, but is *necessary* in order to obtain the energy and building blocks for multicellular development. Indeed, multicellular development does not proceed except under starvation conditions.

In starving *E. coli* cells, COWIE *et al.*[16] have shown that at least a fraction of the ethanol soluble protein is degraded to amino acids which are incorporated into the residual, ethanol insoluble protein. The soluble protein is "expendable" in the sense that it can be used, without impairing cell viability, to maintain the residual protein under adverse conditions. In the slime molds, too, ethanol soluble protein is apparently used first and, percentagewise, to a greater extent. The next paper in this series will demonstrate that the rate of incorporation of [^{35}S]methionine into ethanol soluble protein is lower than that of the ethanol insoluble protein. Perhaps this relative lack of replenishment accounts at least in part for the early and rapid loss of ethanol soluble protein.

Our data are in general agreement with those of GREGG AND BRONSWEIG on net loss of protein during development[4]. They analysed three stages of differentiation, and found a somewhat greater protein utilization between the pseudoplasmodium and fruit than between the myxamoebae and pseudoplasmodium stages.

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PROTEIN AND AMINO ACID TURNOVER DURING DIFFERENTIATION IN THE SLIME MOLD

II. INCORPORATION OF [^{35}S]METHIONINE INTO THE AMINO ACID POOL AND INTO PROTEIN

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SUMMARY

In the slime mold, the size of the endogenous "free" methionine pool is a function only of the stage of development, and is uninfluenced by exogenous methionine. However, exogenous [^{35}S]methionine can exchange with endogenous methionine, and the extent of this exchange (*i.e.*, the specific radioactivity of pool methionine) is a linear function of the exogenous [^{35}S]methionine concentration. The manner in which cellular pool methionine molecules are utilized for protein synthesis is complex; a possible interpretation is suggested in DISCUSSION.

Active protein turnover occurs during differentiation in the slime mold. Methionine protein is replaced by endogenous pool [^{35}S]methionine at the rate of about 7 %/h at preculmination. The rate of protein synthesis appears to decrease slightly during development. Subsequent to a "pulse" of [^{35}S]methionine, the specific radioactivity of various protein classes can differ as much as three-fold. The relative rates of [^{35}S]-methionine incorporation into ethanol soluble as compared to ethanol insoluble protein changes during the course of differentiation.

INTRODUCTION

The results of previous studies on the activity of various enzyme proteins during development necessitated an investigation of the general characteristics of the amino acid pool and of protein synthesis in the slime mold. Experiments designed for this purpose have revealed some unusual properties of the endogenous amino acid pool and of protein synthesis in this differentiating system.

It will be shown that active protein turnover occurs during multicellular development in the starving slime mold. The present study demonstrates that the amount of endogenous methionine per cell is a function of the stage of differentiation. At no stage of development can increasing concentrations of exogenous methionine alter the *size* of the endogenous methionine pool, although increasing concentrations do enhance the ***rate of exchange*** of the pool with the exogenous (labeled) methionine. Evidence will be presented suggesting that these exchangeable methionine molecules extracted by cold trichloracetic acid (TCA), may be qualitatively different with respect to protein synthesis.

MATERIALS AND METHODS

L-[^{35}S]*methionine* was obtained from Abbott Laboratories. The [^{14}C]amino acid mixture was prepared from an algae hydrolysate by Dr. R. HENDLER.

Bonner's salt solution contains in 1 l: NaCl, 0.6 g; KCl, 0.75 g; $CaCl_2$, 0.30 g (see ref. 1).

Dictyostelium discoideum: the myxamoebae were grown with *E. coli* strain ML 304d on a rich medium, washed and plated on purified agar[2] prior to studies on [^{35}S]methionine incorporation.

Methionine was assayed quantitatively according to the procedure described previously[3].

Protein was measured with the biuret method[4] or the absorption at 280 mμ and 260 mμ (see ref. 5).

Protein purification, prior to specific radioactivity determinations, was carried out by the following procedure: (a) precipitation of the total cellular protein by the addition of an equal volume of 6 % TCA, followed by centrifugation and the addition of 6 % TCA to the precipitate, and by boiling at 100° for 15 min; (b) dissolving the protein in 0.5 *N* NaOH, centrifuging and discarding any insoluble material; (c) reprecipitation with 12 % TCA and washing once with 6 % TCA; (d) dissolving the protein in 0.5 *N* NaOH, for quantitative protein and radioactivity determinations. The latter were corrected for self-absorption due to the presence of the alkali. Further purification did not alter the specific radioactivity of the protein samples. When ethanol soluble protein was to be purified (for comparison in specific radioactivity to the ethanol insoluble protein from the same cells), the extraction with 2.0 ml of 75 % ethanol at 45° was carried out immediately following precipitation of the total cellular protein by cold 6 % TCA. After reprecipitation from the ethanol by 12 % TCA, the ethanol soluble protein was carried through steps b–d. Before ethanol insoluble protein was carried through these steps, it was boiled 15 min at 100° in 6 % TCA.

Methionine isolation from protein was carried out as follows: the sample was hydrolysed in 6 *N* HCl in a sealed tube for 4 h at 105° (see ref. 6) . After removing excess HCl by repeated evaporation with heat, the hydrolysate was chromotographed on Whatman No. 1 paper using a solvent (*tert*-butanol–methyl-ethyl ketone–water–diethyl amine (40:40:20:4)) which separates methionine completely from the other major radioactive component found to be present. The latter is not cysteine, and is presumably an oxidation product of methionine[6]. The methionine area (R_F, 0.5–0.6) was eluted for quantitative microbiological assay and radioactivity measurement.

Methionine isolation from solution: A TCA cell extract containing free methionine was passed through a Dowex 50 H^+ (200–400 mesh X 12 cross linked) resin contained in a small tube (1 cm diameter × 10 cm length). The resin (about 1 ml) was washed with 10 ml of 0.01 *M* HCl, followed by 5 ml of water, and the amino acids were eluted quantitatively with 10 ml of 1 *M* NH_4OH. The sample was evaporated to dryness and dissolved in water for further analysis.

RESULTS

Properties of the cellular methionine pool

Kinetics of incorporation of [^{35}S]methionine: The labeling of the amino acid pool and of total cellular protein was followed as a function of time. Cells were grown and plated on washed agar in the usual manner. At late aggregation, 5.0 ml of Bonner's salt solution containing 0.3 μmoles of [^{35}S]methionine ($1 \cdot 10^6$ counts/min/μmole) were added to each of 12 plates. At 15, 60, 90 and 120 min thereafter the cells from groups of three plates were harvested, washed once with Bonner's salt solution containing unlabeled methionine and once with the salt solution alone*. Each sample was frozen until the completion of the experiment, at which time three types of analysis were made on each sample: (a) The size of the methionine pool (total μmoles methionine per three plates in the cold TCA extract[3]). (b) The specific radioactivity of the amino acid pool methionine and (c) the specific radioactivity of the total cellular protein. The data are given in Fig. 1.

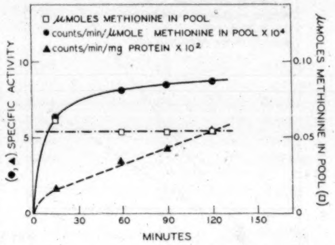

Fig. 1. The labeling of the amino acid pool and of total cellular protein as a function of time. The specific radioactivity of exogenous [^{35}S]methionine was $1 \cdot 10^6$ counts/min/μmole See text for details.

The size of the amino acid pool did not change during the course of this experiment (although during the longer course of differentiation it decreases strikingly in size[3]). The greatest increase in specific radioactivity of the amino acid pool occurs in the first 15 min, and the maximum specific radioactivity of pool methionine attained at equilibrium is about $8 \cdot 10^4$ counts/min/μmole, which is an order of magnitude lower than that of the exogenous methionine ($1 \cdot 10^6$ counts/min/μmole). As will be seen below, only at preculmination does the pool methionine approach the external methionine in specific radioactivity. A kinetic picture similar to the one summarized by Fig. 1 is also observed at other stages of development, as shown in Table I.

Incorporation at different [^{35}S]methionine concentrations: Experiments were carried out in which the incorporation of [S^{35}]methionine into the amino acid pool and into protein was studied as a function of the concentration of externally supplied [^{35}S]methionine of constant specific radioactivity. In the experiment summarized by Fig. 2, carried out at the amoeba stage, the cells were harvested at 45 min, at which time the free amino acid pool had attained its maximal specific radioactivity for each external methionine concentration. A surprising result is the fact that, in contrast to many other microorganisms, the size of the cellular methionine pool is independent of exogenous methionine concentration over the range investigated (0.04–0.32 μmoles/ml). Nevertheless, the specific radioactivity (*i.e.*, the extent of exchange between exogenous and endogenous methionine) of the methionine pool increases linearly with increasing external methionine concentration, although at this stage of development it never attains the specific radioactivity of the exogenous methionine. The curve for protein specific activity does not increase linearly, but tends to fall off at higher exogenous methionine concentrations. This phenomenon will be discussed below in the section dealing with protein synthesis.

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* Further washing does not further decrease the specific activity of the methionine in the amino acid pool, demonstrating the absence of contaminating exogenous methionine which has, relatively, a much higher specific activity (see below).

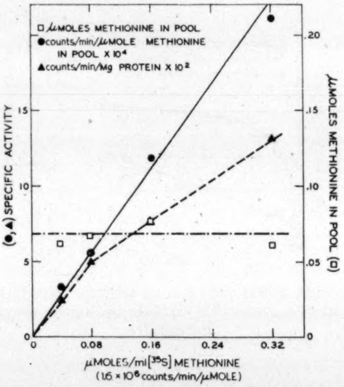

Fig. 2. Incorporation of [^{35}S]methionine into the amino acid pool and into protein as a function of the concentration of exogenous [^{35}S]methionine at constant specific radioactivity. See text for details.

Data are presented in Table I confirming and extending the two main results summarized by Figs. 1 and 2: (a) For a given exogenous [^{35}S]methionine concentration, the maximum specific radioactivity of the methionine pool is attained in about 20 min, and this value is less than the exogenous methionine specific radioactivity; (b) Increasing the concentration of exogenous [^{35}S]methionine does not "expand"[7] the methionine pool, but does increase its specific radioactivity. Table I also illustrates the fact that the size of the amino acid pool as well as the exchangeability of its amino acids is dependent upon the stage of differentiation.

At the three stages of development indicated in Table I, cells were exposed for 15 or 45 min to two different exogenous [^{35}S]methionine concentrations. At 15 and 45 min, for each methionine concentration, cells were harvested and washed in the manner previously described. For each sample, the absolute amount of methionine in a (comparable) cell aliquot was determined, as was the specific radioactivity of the amino acid pool and total cellular protein. It can be seen that the size of the methionine pool drops during differentiation, and that it is never increased by a ten fold increase in exogenous methionine concentration. For each stage of development the maximal specific radioactivity which is attained in 15 min is a linear function of exogenous methionine concentration, and therefore increases 10 fold from the lowest to the highest exogenous [^{35}S]methionine concentration (see also Fig. 2). Note that, for a given exogenous methionine concentration, the specific radioactivity (exchangeability) of the endogenous methionine pool is maximal at the slug stage. Other experiments demonstrate that maximal exchangeability actually occurs at preculmination.

TABLE I

SIZE AND SPECIFIC RADIOACTIVITY OF THE CELLULAR METHIONINE POOL AS A FUNCTION OF (a) THE STAGE OF DIFFERENTIATION AND (b) EXOGENOUS [^{35}S] METHIONINE CONCENTRATION

Stage	*Exogenous [^{35}S]methionine μmoles/ml*	*Time exposed to [^{35}S]methionine (min)*	*Total μmoles methionine in cells*	*Counts/min/μmole methionine in cells × 10^6*
Amoeba	0.04	15	0.093	0.0156
	0.04	45	0.105	0.0130
Amoeba	0.40	15	0.114	0.123
	0.40	45	0.096	0.144
Slug	0.04	15	0.021	0.125
	0.04	45	0.015	0.116
Slug	0.40	15	0.017	0.913
	0.40	45	0.016	1.210
Fruit	0.04	15	0.022	0.033
	0.04	45	0.021	0.056
Fruit	0.40	15	0.020	0.415
	0.40	45	0.020	0.487

In order to obtain some preliminary information concerning the mechanism of exchange between the amino acid pool and exogenous methionine, the effect of 2,4-dinitrophenol was studied. Table II demonstrates that the exchange reaction (as well as protein synthesis) is inhibited about 50% at $4 \cdot 10^{-4}$ M 2,4-dinitrophenol, suggesting an energy-dependent process.

TABLE II

EFFECT OF 2,4-DNP ON POOL EXCHANGE AND PROTEIN SYNTHESIS AT PRECULMINATION

Concn. 2,4-DNP	*% inhibition*	
	Exchange	*Protein*
$2 \cdot 10^{-4}$	34	32
$4 \cdot 10^{-4}$	55	47

Having thus established certain properties of the endogenous methionine pool of the slime mold, it is now possible to examine the characteristics of protein synthesis from this amino acid pool during differentiation.

Protein synthesis from the cellular methionine pool

Preliminary to studies on the rate of protein synthesis, it was necessary to establish that, at any stage of development, the specific radioactivity of total cellular protein truly reflects the rate of replacement of protein methionine, and not, for example, the relative abundance of methionine-rich proteins. Therefore, the specific radioactivity of protein labeled at various stages of development was compared with the specific radioactivity of the methionine isolated from this protein. At the amoeba, slug, preculmination and fruit stages, cells were exposed to $[^{35}S]$methionine for 3 h in Bonner's salt solution. Incorporation of $[^{35}]$Smethionine into protein is linear after the first 15 min (Fig. 1). The total cellular protein was then isolated, purified, and its specific radioactivity determined. These values are represented by the solid circles in Fig. 3. An aliquot of each protein sample was then hydrolyzed and the specific radioactivity of the isolated methionine determined. It can be seen that the ratio of these two specific radioactivities is constant, in spite of the fact that at preculmination the values are four-fold those at the amoeba stage. We may conclude that at all stages of development, on the average, protein specific radioactivity truly reflects methionine specific radioactivity, and that the latter can be obtained from the former by applying a factor of 12.5 $\left(= \frac{\text{methione specific radioactivity}}{\text{protein specific radioactivity}}\right)$

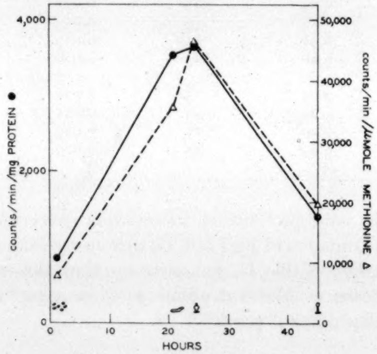

Fig. 3. The relationship of protein specific radioactivity to protein-methionine specific radioactivity, following a 3-h exposure to $[^{35}S]$methionine at 4 stages of differentiation.

It would appear from these data that the rate of protein synthesis increased almost one order of magnitude during early differentiation. We shall see below that, instead, the extent of labeling the amino acid pool increased, while the rate of protein synthesis from this pool if anything decreased.

Incorporation into protein at different $[^{35}S]$*methionine concentrations*: Although cellular pool methionine specific radioactivity is a linear function of exogenous $[^{35}S]$ methionine concentration, this is not true of protein specific radioactivity. The data of Fig. 2 show the tendency of the protein curve to fall off from linearity as the exogenous $[^{35}S]$methionine concentration increases. Table III summarizes two other experiments in which the protein specific radioactivity failed to increase in parallel with the pool specific radioactivity, in response to a tenfold rise in exogenous $[^{35}S]$-methionine concentration. Thus the apparent rate of protein synthesis from the amino acid pool (% incorporation of [^{35}S]methionine—last column) decreases as the specific radioactivity of the amino acid pool increases (see calculations below).

TABLE III

INCORPORATION OF [^{35}S]METHIONINE INTO PROTEIN

Expt.	*Stage*	*Exogenous [^{35}S]methionine µmoles/ml*	*Average counts/min/µmole pool methionine/30 min × 10^6*	*Counts/min/mg protein Δ/30 min**	*%** incorporation*
I	Amoebae	0.04	0.018	134	9.2
		0.40	0.11	660	7.6
	Slug	0.04	0.24	1,144	5.9
		0.40	2.10	6,360	3.7
II	Amoebae	0.04	0.014	127	11.1
		0.40	0.133	840	7.9
	Slug	0.04	0.120	1,282	14.4
		0.40	1.061	5,040	6.2

* 45 min value minus 15 min value.

** $\% \text{ incorporation} = \dfrac{\Delta \text{ protein specific act.} \times 12.5 \times 100}{\text{average pool methionine specific act.}}$

Calculation of the rate of protein synthesis: The formula used for calculating % incorporation is: $\% \text{ incorporation} = \dfrac{\Delta \text{ protein specific act.} \times 12.5}{\text{average pool methionine specific act}} \times 100.$

It is of course necessary to calculate the % incorporation of methionine into protein during a time period in which the pool methionine specific radioactivity is constant. Thus after optimal pool methionine specific radioactivity is attained, two values, at 15 and 45 min, are determined and averaged. Over this same time period (30 min) the increase in protein specific radioactivity is determined.

For example, in the case of the amoebae at 0.04 µmoles methionine/ml (Table III Expt. II), the increase in protein specific radioactivity, 127 counts/min, is multiplied by 12.5, (which is the ratio of protein methionine specific radioact./protein specific radioact.) thus converting it to counts/min/µmole methionine (see relationship of protein specific radioactivity to methionine specific radioactivity, Fig. 3). This value, 1,587, is divided by the average specific radioactivity of the amino acid pool over the 30-min period ($0.014 \cdot 10^6$ counts/min/µmole), giving the percent incorporation per 30 min period as 11.1 %. At the high exogenous [^{35}S]methionine concentration the rate of incorporation has decreased to 7.9 %.

There are two general explanations which could account for the (apparent or genuine) decrease in rate of protein synthesis at higher pool specific radioactivities: (a) High exogenous methionine concentrations inhibit protein synthesis. (b) The pool methionine molecules are not equivalent with respect to protein synthesis: *on the average*, the methionine molecules in the amino acid pool labeled at low pool specific radioactivities became incorporated into proteins attaining a relatively high specific radioactivity; pool methionine molecules labeled at high pool specific radioactivity become incorporated into proteins attaining a relatively lower specific radioactivity.

Effect of high exogenous methionine on protein synthesis: In order to test directly whether a high exogenous methionine concentration inhibits protein synthesis, the following experiment was carried out:

Cells at late aggregation were incubated in Bonner's salt solution for 7 min in the absence (A) and presence (B) of a mixture of [^{14}C]amino acids to label the amino acid pool. Each cell suspension was quickly centrifuged, and the unlabeled cells suspended in a salt–[^{14}C]amino acid mixture. Half of these cells were frozen immediately, and half were frozen after an additional 9-min incubation. The cells previously labeled (B) were divided and incubated in the absence (B1) and presence (B2) of unlabeled methionine (0.3 μmoles/ml). In both these cases, samples were again frozen at zero and 9 min. As can be seen from Table IV experiment A, active incorporation of exogenous [^{14}C]amino acids was occurring during the 9-min incubation period. The 0 min sample of Expt. A could not be frozen quickly enough to prevent all incorporation. Expts. B1 and B2 demonstrate that protein synthesis from [^{14}C]amino acids within the cells is not inhibited by a high concentration of unlabeled exogenous L-methionine.

TABLE IV

EFFECT OF EXOGENOUS METHIONINE ON PROTEIN SYNTHESIS

Expt.	*Additions*		*Counts/min/mg protein*		
	7 min preincubation	*9 min incubation*	*0 min*	*9 min*	*Δ*
A	None	[^{14}C]amino acids	272	625	353
B1	[^{14}C]amino acids	None	737	1,180	443
B2	[^{14}C]amino acids	Unlabeled methionine	777	1,187	410

We are thus left with the alternative general explanation that, at higher pool specific radioactivities (compared to lower pool specific radioactivities), relatively more labeled methionine molecules are incorporated into proteins of low specific radioactivity. We have in fact found clear heterogeneity in the specific radioactivities of various arbitrary classes of slime mold protein following a pulse of [^{35}S]methionine.

Heterogeneity in specific radioactivities of different protein classes: We have seen previously (Fig. 1) that incorporation of [^{35}S]methionine into total cellular protein is linear after the first 15 min. Specific radioactivities of ethanol soluble and ethanol insoluble protein were also followed as a function of time of exposure to exogenous [^{35}S]methionine. This experiment was carried out at preculmination, since the difference in the rate of incorporation of [^{35}S]methionine into these two classes of proteins is usually greatest at this stage of development (Fig. 4).

In this experiment, there is about a three-fold difference in the specific radioactivity of these two classes of proteins. The extent of this difference varies from experiment to experiment; the variables involved are not yet understood. Table V gives the ratio of specific radioactivities of these two protein fractions as a function of the stage of differentiation. At each of the stages indicated, the cells were exposed to [^{35}S] methionine (0.2 μmoles/ml) for 1 h, harvested, and the two types of protein purified for specific radioactivity determinations. Thus the specific radioactivity of ethanol insoluble protein is higher than that of ethanol soluble protein at all stages of development, and this difference becomes maximal at preculmination.

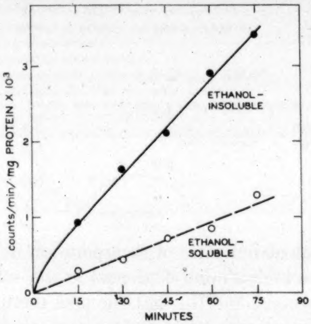

Fig. 4. The specific radioactivity of ethanol soluble and ethanol insoluble protein at preculmination as a function of time.

TABLE V

SPECIFIC RADIOACTIVITIES OF TWO CLASSES OF PROTEINS AS A FUNCTION OF THE STAGE OF DEVELOPMENT

Stage	*Specific radioactivity:* *ethanol insoluble / ethanol soluble* *(counts/min/mg protein)*
Amoeba	1.56
Aggregation	2.11
Early slug	2.27
Slug	2.70
Preculmination	4.15
Fruit	2.82

These changes in ratio observed as a function of differentiation may be due either to (a) differential rates of synthesis of the two classes of protein, or (b) differential rates of utilization of methionine rich (or poor) protein. Both phenomena could of course be involved.

In order to observe the specific radioactivities of many more classes of protein following a pulse of [^{35}S]methionine, DEAE column chromatography was used. The various protein classes resolved by this procedure showed heterogeneity in specific radioactivities, with maximal differences of about three-fold[8]. It is clear from these experiments that significant variations in protein specific radioactivities exist.

The rate of protein synthesis at different stages of development: A summary of calculated rates of methionine incorporation at various stages of development and at various external methionine concentration is presented in Table VI.

At any particular stage of development the rate of protein synthesis appears to decrease at relatively higher exogenous methionine concentrations (Table III). However, it can be seen from Table VI that comparing what seem to be similar stages in different experiments can give quite variable absolute rates of synthesis.

TABLE VI

% INCORPORATION PER 30 min INTO PROTEIN

Experiment	*I*	*II*	*III*	*IV*	*V*	*VI*
µmoles [^{35}S]methionine/ml	0.04	0.04	0.10	0.20	0.40	0.40
Amoebae	9.2	11.1			7.6	7.9
Blob			4.0	4.5		
Slug	5.9	14.4			3.7	6.2
Early preculmination			4.1	3.4		
Late preculmination				2.9		
Young fruit		3.9			4.4	
Old fruit			10.7	6.6		

The validity of the calculated rates of incorporation depend upon two assumptions: (a) That the TCA-soluble amino acid pool is the sole source of amino acid precursors for protein synthesis, and (b) that the pool methionine molecules labeled at all external [^{35}S] methionine concentrations are equivalent with respect to protein synthesis. That this second assumption may not be entirely correct appears evident from the results presented previously.

Since our data have shown that pool methionine molecules are heterogeneous with respect to their rate of exchange, it may not be possible to observe the "true" rate of their incorporation into protein except when complete exchange of external and pool methionine has occurred. This situation can at least be closely approximated at high exogenous methionine concentrations during preculmination, which is metabolically the most active stage of development. From Table VI it can be seen that, at the higher exogenous methionine concentrations during early and late preculmination (Expts. III and IV). the incorporation rate was between 2.9 and 4.1 %/30-min period, or about 7 %/h. In these cases, the specific activity of the amino acid pool was more than half that of the exogenous methionine. The data in this table, summarizing six experiments, suggest that the overall rate of protein synthesis decreases slightly during development and is minimal at preculmination. However, this decrease may be only apparent, if the relatively small fraction of pool methionine molecules which can be labeled in the early stages of development are not representative of the higher percentage of labeled molecules at preculmination.

DISCUSSION

In order to facilitate the discussion, a schematic description of some of the biochemical events occurring during differentiation in the slime mold is presented in Fig. 5. The first four items listed in the diagram are dealt with in other publications[2,3,9,10].

Because of the indicated increase in the rate of exchange of "free" amino acids in the cells, the pool and hence protein specific activities increase at the beginning and then decrease towards the end of differentiation. The increased rate of exchange of pool amino acids may in part be due to a decrease in the absolute size of the pool and may in part be a reflection of turnover: their rate of formation from protein breakdown and their rate of utilization. Since the rate of protein synthesis does not increase simultaneously with cellular amino acid turnover, it is quite possible that the latter increases from enhanced net protein breakdown coupled with intensive carbohydrate synthesis from the resulting amino acids. The enhanced activity of these two metabolic processes at preculmination is well documented[3, 9, 10].

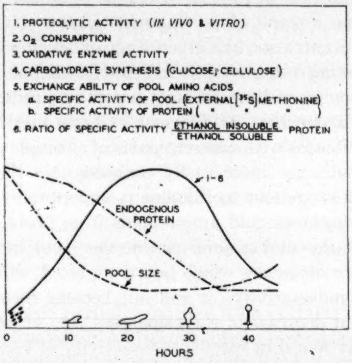

Fig. 5. A schematic representation of various biochemical changes occurring during differentiation in the slime mold. For further explanations, see text.

At preculmination, when the pool specific activity is almost as high as that of the exogenous [^{35}S]methionine, the rate of protein synthesis is about 7 %/h. This value can be compared to that of resting bacteria, 5 %/h[11], and that of animal cells in tissue culture, 1 %/h[12].

Judging from the behavior of methionine, and from total pool amino acid determinations[3], the size of the amino acid pool is a function only of the stage of development, uninfluenced by exogenous amino acids. This is significant in view of the requirement of starvation for the initiation of multicellular differentiation. Thus the slime mold cells have a barrier to concentrating at least one amino acid, although an exchange with existing molecules can occur. An analogy to this situation may be found in the exchange-diffusion of phosphate across cell walls[13]. Manometric studies indicate no oxidation (and hence no net uptake?) of exogenous methionine or serine[14], and determinations of changes in cell volume suggest that glycine and cysteine do accumulate inside the cell whereas arginine does not[15]. It is also clear that exogenous histidine accumulates in the cell[18].

In all probability, the cellular amino acid pool is the source of precursors for protein synthesis, particularly since the slime mold normally depends only on endogenous amino acids. Investigations in *E. coli* and in yeast demonstrate that externally-supplied amino acids must pass through a cellular pool prior to incorporation into protein[16, 17*].

Assuming that only endogenous amino acids are used for protein synthesis, it would seem unlikely that increasing the exogenous methionine concentration tenfold could inhibit general protein synthesis by 50 %, particularly since the endogenous methionine pool is not expandable (Table I). Moreover, an experiment specifically

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[*] If the rate of protein synthesis in the slime mold were calculated from the specific activity of exogenous methionine the rate would increase tenfold in going from the amoebae to the slug stage in Table III, Expt. I.

designed to test whether high exogenous methionine concentrations inhibit protein synthesis was negative (Table IV). In view of these considerations, it is felt that the decrease in the calculated rate of protein synthesis obtained when the pool specific radioactivity increases (as a result of development or as a result of increasing exogenous [^{35}S]methionine concentration at a given stage of development) is only apparent, and not real. The following tentative explanation is offered for this phenomenon: the fact that the methionine of the pool does not fully equilibrate with that of the external methionine indicates that, at different external methionine concentrations, labeled methionine equilibrates with different parts of a complex pool. In other words, some methionine molecules are more easily exchangeable than others[7]. (This lack of full equilibration with exogenous methionine is undoubtedly in part due to a high rate of formation of endogenous cold amino acids from protein catabolism.) As the specific radioactivity of the endogenous methionine pool increases, an increasing number of the methionine molecules which become labeled will be incorporated into proteins of low specific radioactivity, or will not become incorporated into protein at all. Hence an apparent decrease in the calculated rate of protein synthesis is observed. The heterogeneity found in specific radioactivity of various classes of protein could be due to (a) different methionine contents or (b) different rates of synthesis. Preliminary experiments support the latter interpretation[8].

If our speculation is valid, and pool amino acids have different fates with respect to protein synthesis, it is conceivable that changing the number or pattern of pool amino acids (*e.g.*, through starvation) could effect changes in the patterns of synthesized protein.

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PURIFICATION AND PROPERTIES OF A LEUKEMIC CELL PHOSPHODIESTERASE

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SUMMARY

A phosphodiesterase has been partially purified from extracts of mouse leukemic cells (L4946) carried as an ascitic tumor. Purified fractions resemble snake venom phosphodiesterase in forming nucleoside 5′-phosphates from RNA, from polymers synthesized with polynucleotide phosphorylase, and from oligonucleotides as small as a dinucleotide. As with venom, the cleavage appears to be stepwise by splitting off a single mononucleotide unit at a time from an end of the chain. However, in contrast to the venom enzyme, the leukemic cell phosphodiesterase is unable to hydrolyze simple benzyl or *p*-nitrophenyl esters of nucleoside 5′-phosphates. It thus belongs to a new class of phosphodiesterases with a specificity requiring that two nucleoside moieties must be in ester linkage to a phosphate group.

The rates of hydrolysis of different polynucleotides differ very widely.

INTRODUCTION

Of the phosphodiesterases which hydrolyze nucleic acids to nucleoside monophosphates, two have been very extensively studied; these are snake venom phosphodiesterase, which hydrolyzes polynucleotides to nucleoside 5′-phosphates, and spleen phosphodiesterase, which forms nucleoside 3′-phosphates. Both of these enzymes also hydrolyze simple esters of appropriate mononucleotides, such as benzyl adenosine 5′-phosphate in the case of the venom enzyme[1] or benzyl adenosine 3′-phosphate for spleen phosphodiesterase[2]. It appears, therefore, that substrates suitable for these enzymes need to contain only a single nucleoside moiety. The relevant literature has been recently reviewed[3].

We now wish to report on a phosphodiesterase partially purified from tumor cells which falls into a new classification. This enzyme hydrolyzes poly A*, poly AU, RNA

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* The system of abbreviations used for oligonucleotides is described in "Use of Abbreviations" in current issues of the *Journal of Biological Chemistry* and in ref. 34. Poly A, poly U, poly C, poly G, poly AU, and poly AGUC are the polymers made with polynucleotide phosphorylase from the corresponding nucleoside 5′-diphosphates. Other abbreviations used are: RNA, ribonucleic acid; 5′-AMP, adenosine 5′-phosphate; 5′-UMP, uridine 5′-phosphate; 5′-IMP, inosine 5′-phosphate; ADP, adenosine diphosphate; UDP, uridine diphosphate; GDP, guanosine diphosphate; ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)-aminomethane-HCl buffer; EDTA, ethylenediaminetetraacetate; DEAE-cellulose, diethylaminoethylcellulose.

(very slowly) and oligonucleotides with a 5′-phosphomonoester end group, giving rise to nucleoside 5′-phosphates. Both ribonucleotides, such as pApA, pApApA and pUpU, and deoxyribonucleotides such as pTpT are hydrolyzed. In these respects, the specificity of the enzyme resembles that of venom phosphodiesterase. However, a difference is found in that simple esters such as benzyl adenosine 5′-phosphate and *p*-nitrophenyl thymidine 5′-phosphate are not split; a substrate must have at least two nucleoside residues.

EXPERIMENTAL

Materials

Poly A, poly U, poly AU, poly C and poly AGUC were synthesized by a published procedure[4] using polynucleotide phosphorylase from *Escherichia coli*[5] or *Azotobacter agilis*[4]. Samples of poly A made with the enzyme from *Micrococcus lysodeikticus*[6] were also used for many of the assays; these were kindly furnished by Dr. R. F. BEERS, Jr., Children's Hospital School, Baltimore, Maryland. Poly G was synthesized with the *A. agilis* enzyme, using pApApA as a primer[7]. Previously published methods were used for the isolation of pApA[8], pApApA[8], pApApApA[8], pApApApApA[8], pUpU[9], pUpUpU[9], ApUp[10], ApApUp[10], ApCp[11], ApA[8], and ApApA[8]. The tetranucleotides pApApApU and pApApApG were isolated after incubating UDP[12] or GDP[7] with pApApA and polynucleotide phosphorylase. The oligodeoxyribonucleotides, d-pTpT, d-pTpTpT, d-pTpTpTpT and d-pApA were chemically synthesized[13,14], and were kindly donated by Drs. G. TENER and H.G. KHORANA, British Columbia Research Council, Vancouver, B.C.

Mononucleotides with a 2′,3′-cyclic phosphoryl group, such as adenosine 2′,3′-phosphate were purchased from Schwarz Laboratories, Mt. Vernon, N. Y. Benzyl cytidine 3′-phosphate and benzyl adenosine 3′-phosphate were chemically synthesized[2,15]. Benzyl adenosine 5′-phosphate[1] was a gift from Dr. D. M. BROWN, The Chemical Laboratory, Cambridge, England. The *p*-nitrophenyl esters of thymidine 5′-phosphate[16], thymidine 3′-phosphate[17] and uridine 5′-phosphate[18] were gifts from Dr. H. G. KHORANA and Dr. W. E. RAZZELL. Highly polymerized RNA isolated from turnip yellow mosaic virus[19] was kindly supplied by Dr. R. MARKHAM, Plant Virus Unit, Agricultural Research Council, Cambridge, England. Other polymer substrates included yeast RNA[19], and bacterial RNA from *A. agilis* kindly furnished by Dr. R. M. S. SMELLIE, Department of Biochemistry, University of Glasgow, Glasgow, Scotland. Purified 5′-nucleotidase was prepared from bull seminal plasma[21]; it was free of phosphodiesterase activity. DEAE-cellulose was purchased from Eastman Kodak Company, Rochester, N.Y. Protamine sulfate was obtained from Eli Lilly and Co., Indianapolis, Indiana.

METHODS

Enzyme assays

Assay of the leukemic cell phosphodiesterase was based on the formation of perchloric acid soluble nucleotides from poly A. The reaction mixture (0.06 ml) contained poly A equivalent to 0.2 μmole of adenine, 0.15 μmole of $MgCl_2$, 0.5 μmole of glutathione, 0.005 μmole of EDTA, 4 μmoles of potassium phosphate buffer, pH 7.6, and from 0.015 to 0.15 unit of enzyme*. The mixture was incubated at 37° for 30 min* and the reaction was terminated by the addition of 0.34 ml of 3 % perchloric acid. After being kept for 10 min at 0°, the mixture was centrifuged at 1500 × *g* for 5 min to remove coagulated protein and poly A. A 0.2-ml aliquot of the supernatant fluid was mixed with 0.8 ml of water and the O.D. was measured at 250 mμ in the Beckman model DU spectrophotometer, using silica cells with a 1-cm light path. This wave length was chosen because it is an isosbestic point for 5′-AMP and 5′-IMP. Both of these products were formed by the crude extracts that contain 5′-AMP deaminase. Control incubations lacking poly A or enzyme were run, and these readings were subtracted from the experimental readings. The blank due to poly A was usually negligible.

Activity toward the *p*-nitrophenyl esters was measured as follows: The reaction mixture (0.125 ml) contained 1 μmole of the ester, 12.5 μmoles of Tris, pH 8.8, 1 μmole of $MgCl_2$, and enzyme. The mixture was incubated at 37° for periods up to 1 h after which 0.475 ml of 0.1 *N* NaOH was added. Any turbidity, if present, was removed by centrifugation at 2,000 × *g* for 2 min, and the O.D. at 400 mμ was measured immediately.

In both assays, one unit of enzyme activity is defined as that amount of enzyme which forms 1 μmole of product/h. Specific activity is calculated as units/mg of protein. Protein was measured by the method of LOWRY *et al*[22].

Chromatography

Descending chromatography on Whatman No. 3MM paper was carried out with the following solvent systems: Solvent 1, *n*-propanol–concentrated NH_4OH–water (60:30:10, v/v/v)[23]; Solvent 2, saturated ammonium sulfate–isopropanol–1 *M* sodium acetate (80:2:18, v/v/v)[24]; Solvent 3, isobutyric acid–1 *M* NH_4OH–0.2 *M* EDTA (100:60:0.8, v/v/v)[25]; Solvent 4, isopropanol–water (70:30, v/v) with NH_3 in the vapor phase[26]. Paper electrophoresis of nucleotides was performed according to MARKHAM AND SMITH[26] on strips (56 cm × 9 cm) of Whatman No. 3MM paper saturated with 0.05 *M* ammonium formate–formic acid buffer, pH 3.5. Purine- and pyrimidine-containing compounds were located on paper with a Mineralight lamp. For quantitative estimation, the compounds were eluted from paper with 0.1 *N* HCl and estimated spectrophotometrically, using appropriate paper blanks. For enzymic identification the materials were eluted with water.

DEAE-cellulose for column chromatography[27] was washed extensively with water and then with a solution of 0.03 *M* Tris, pH 7.2, that also contained 0.001 *M* EDTA and 0.005 *M* β-mercaptoethanol[28].

RESULTS

Purification of the leukemic cell phosphodiesterase

Mouse lymphocytic leukemia, L4946, in ascitic form, was originally obtained from Dr. LLOYD LAW of the National Cancer Institute and Dr. R. JORDAN of the City of Hope Medical Centre; both of these lines gave similar results. The tumor was carried, and harvests grown, in $(C_3H \times AKR)F_1$ hybrid mice[29]. Cell growth from an inoculum of $2 \cdot 10^6$ cells was harvested after 6 days. The ascites cells were washed with 0.9 % NaCl and disrupted in 0.1 *M* Tris, pH 7.3, by means of high frequency vibration, using stainless steel beads and a Syntron paper jogger (Type PI3, Style 1783, from Syntron Co., Homer City, Penn.). All steps in the fractionation were done at 3° except as stated. The mixture was centrifuged at 15,000 × *g* for 10 min and the precipitate discarded. The crude extract was diluted with 0.1 *M* Tris, pH 7.3, to a final volume equal to 3 times the volume of the packed ascites cells, and β-mercaptoethanol was added to a concentration of 0.005 *M* (crude extract, 100 ml, Table I). The pH was adjusted to pH 6.9 with 2 ml of 0.1 *M* sodium acetate–acetic acid buffer, pH 3.5 and the extract was heated for 10 min at 53°. The mixture was immediately cooled to 3°, and a precipitate was removed by centrifugation at 15,000 × *g* for 3 min. This step resulted in some loss of activity, but gave a 2.5-fold purification and resulted in complete destruction of contaminating adenosine 5′-phosphate deaminase.

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* For most fractions, the extent of reaction was proportional to amount of enzyme between these limits which correspond to 3–30 % hydrolysis. With heated crude extracts, however, the range of proportionality was more narrow. The reaction was also linear with time up to 30 % hydrolysis.

TABLE I

PURIFICATION OF THE PHOSPHODIESTERASE FROM CELLS OF MOUSE LEUKEMIA L4946

Fraction	*Volume of fraction (ml)*	*Total activity (units)*	*Overall yield (%)*	*Specific activity units/mg protein*
Crude extract	100	700	100	1.5[*]
Heated extract	93	575	82	3.6
Supernatant from Protamine I	93	585	84	3.9
Eluate from Protamine II	37	520	74[**]	15.5

[*] Crude extracts vary in specific activity from this level up to 4 units/mg protein; from more active extracts final protamine eluates with specific activities up to 27 were prepared.

[**] Final yields were frequently below this figure and vary from 30 % to 75 %.

To the supernatant solution (heated extract, 93 ml, Table I) was slowly added 4.2 ml of 1 % protamine sulfate. The mixture was kept at 3° for 15 min, with occasional stirring. An inactive precipitate was removed by centrifuging for about 1 min at 15,000 × *g*[*]. The supernatant solution (protamine I, 93 ml, Table I) was diluted 6-fold with cold distilled water. Enough additional β-mercaptoethanol was added to make the final concentration 0.0025 *M*, and the pH was adjusted to pH 7.4 with 0.2 *N* NH_4OH.

To this solution was slowly added 3.16 ml of 1 % protamine sulfate. After 10 min at 3° with occasional stirring, the resulting precipitate was collected by centrifugation as above, and the supernatant solution was discarded. The tubes were allowed to drain briefly (1–2 min), and the precipitate was disrupted with a glass rod and mixed with 0.2 *M* phosphate buffer, pH 7.2, containing 0.005 *M* β-mercaptoethanol. The volume of eluting buffer was 0.4 that of the protamine I fraction. The mixture was centrifuged as before and the supernatant solution collected (Protamine II, 37 ml,

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[*] This step accomplished little purification but did appreciably reduce the blank absorption at 250 mμ, thereby markedly increasing the sensitivity of the assay.

Table I)[*]. Except where otherwise specified, this fraction was used for the studies reported in this paper.

As shown in Table I, this procedure gave a 10-fold purification. Further, it resulted in complete removal of phosphomonoesterase and adenosine $5'$-phosphate deaminase activities. All efforts to obtain further purification by the older methods of fractionation were unsuccessful. However, a preparation with a specific activity about 3-fold greater than that of the protamine eluate was obtained by DEAE-cellulose chromatography.

Heated extract, prepared as described above, was diluted 2-fold with cold distilled water and applied to a DEAE-cellulose column. Elution was carried out with a gradient of 0 to 0.5 M NaCl in 0.03 M Tris–0.001 M EDTA–0.005 M β-mercaptoethanol. Certain experimental details and the results are presented in Table II. Active protein with approximately the same specific activity as the protamine eluate was readily eluted in a single peak. However, by pooling fractions from the centre of the peak, a preparation of greater purity but smaller yield could be obtained (Table II).

TABLE II

PURIFICATION OF LEUKEMIC CELL PHOSPHODIESTERASE BY CHROMATOGRAPHY ON DEAE-CELLULOSE

Experiment No.	*Fraction*	*Total activity units*	*Overall yield %*	*Specific activity units/mg protein*
1	Heated extract (3.75 ml)[*]	48.6	100	5.4
	DEAE eluate[**]			
	Tubes 12–18, 18 ml	24.5	50.5	—
	Tubes 14–15, 5.5 ml	14.3	29.4	63
2	Heated extract (9.5 ml)[*]	125	100	4.5
	DEAE eluate[***]			
	Tubes 15–21, 50 ml	75.1	60	15.5
	Tube 20, 5.2 ml	18.7	15	51

[*] These heated extracts are comparable to those used for further purification with protamine (Table I).

[**] For this experiment, the mixing chamber contained 52.5 ml of 0.03 M Tris, pH 7.2, 0.001 M with respect to EDTA and 0.005 M with mercaptoethanol. The reservoir solution (52.5 ml) was similar in composition except that it also contained 0.5 M NaCl. The dimensions of the DEAE column were 0.9 × 9 cm and the flow rate was 1 ml/min. Tube 12 was collected after 17.2 ml had passed through the column and tube 14 after 23.2 ml.

[***] Conditions were similar except that the reservoir and mixing chamber each contained 127 ml, the column measured 2.2 × 11 cm, and flow rate was 2 ml/min. Tube 15 was collected after 104 ml and tube 20 after 144 ml.

Properties of the enzyme

pH optimum and the effect of sulfhydryl reagents and of various ions: The enzyme has a rather broad pH optimum between pH 7 and pH 8 (Fig. 1). The reaction is stimulated to a variable extent by sulfhydryl reagents; β-mercaptoethanol or glutathione was, therefore, routinely used to stabilize the enzyme during purification and storage. The hydrolysis of poly A requires Mg^{++}ions; the optimal concentration is $3 \cdot 10^{-3}$ M

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[*] This step usually gave satisfactory results, but did, upon occasion, produce a fraction which for unknown reasons, was distinctly lower in specific activity than the usual product.

and concentrations 3 times as high or higher are inhibitory. This inhibition is not observed in the hydrolysis of oligonucleotides, such as pApA and pApApA and with such compounds good activity is observed with concentrations as high as $8 \cdot 10^{-3}$ *M* Mg^{++}.

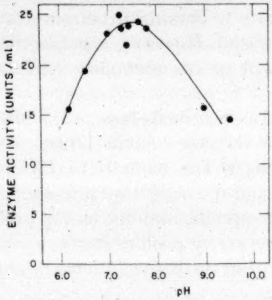

Fig. 1. Effect of pH on the rate of hydrolysis of poly A by purified leukemic cell phosphodiesterase. The assay was carried out as described under METHODS, but using phosphate buffers at a final concentration of 0.09 *M* for pH values up to 7.8. For the value at pH 9 the reaction mixture contained 0.1 *M* Tris, and for pH 9.5, 0.1 *M* glycine. The pH values are those of the final reaction mixture. The enzyme fraction was the protamine eluate (Table I).

At $3 \cdot 10^{-3}$ *M*, Mn^{++} stimulates the hydrolysis of poly A two-thirds as much as Mg^{++}, and Ca^{++} is even less effective. The effect of several other ions on the hydrolysis of poly A was tested under routine assay conditions, except that the concentration of $MgCl_2$ was 0.008 *M*. A 40 % inhibition is found with 0.01 *M* inorganic pyrophosphate and complete inhibition with 0.04 *M* fluoride. A 4-fold stimulation results from the addition of 0.04 *M* phosphate; this is not found with oligonucleotide substrates, and probably represents an interaction involving poly A and both phosphate and Mg^{++}.

Stability: Crude extracts of the ascites cells can be stored for at least 6 months at —15° without significant loss in activity. Heated extracts occasionally show some loss after several days. The protamine eluate (Table I) usually shows no loss after 2 months at —15°, but in several instances up to 50 % loss of activity has occurred. Fractions from DEAE-cellulose are very unstable as eluted from the columns, but they can be stored for at least 3 weeks if they are concentrated by ammonium sulfate precipitation, and the precipitate redissolved in one-fifteenth of the original volume of a solution containing 0.04 *M* Tris, pH 7.2, 0.004 *M* β-mercaptoethanol, 0.001 *M* EDTA and 25 % glycerol.

Heating to 50–55° for 10 min at pH 6.9 results in loss of one-third of the activity (Table I). Heating at a lower pH (5.5 to 6.2) or at a higher temperature (57°–59°) produces significantly more inactivation.

Characteristics of the hydrolysis of poly A

A substrate saturation curve for poly A shows K_m to be approx. $2.0 \cdot 10^{-3}$ *M*, expressed as adenine equivalents, and substrate saturation is achieved at a concentration of $5.0 \cdot 10^{-3}$ *M*. These values are not affected by varying the concentration of Mg^{++} from $2 \cdot 10^{-3}$ *M* to $8 \cdot 10^{-3}$ *M*. The hydrolysis of poly A is proportional to amount of enzyme only up to the point at which $1 \cdot 10^{-3}$ *M* 5′-AMP is formed, corresponding to 30 % hydrolysis in the standard assay. With more enzyme, very little additional reaction occurs. The hydrolysis of poly A is linear with time, but only as long as the concentration of 5′-AMP formed in the reaction is less than 1 to 1.5 μmoles/ml. Thereafter the time curve is almost flat. No satisfactory explanation for this behavior is available, for 5′-AMP at this concentration does not inhibit the enzyme (Table III). The hydrolysis of poly A is inhibited by oligonucleotides such as pApA (Table III), but these could not be detected as intermediates. It is possible that comparatively large polynucleotide fragments are formed from poly A and that such intermediates have a high affinity for the enzyme but undergo very slow further hydrolysis.

TABLE III

INHIBITION OF THE HYDROLYSIS OF POLY A

Routine assay conditions were used, as described under METHODS. The enzyme fraction was a protamine supernatant (Table I) for the experiments with 5′-AMP and adenosine, and a protamine eluate for the experiments with pApA.

Addition	*Concentration μmoles/ml*	*Inhibition %*
5′-AMP	1.4	0
5′-AMP	2.8	30
Adenosine	1.9	70
pApA	0.4	6
pApA	1.6	62

Hydrolysis of other polymers and of oligonucleotides

Poly A is hydrolyzed more rapidly than any of the other polymers in the series. Poly U, poly G and poly AU are split about one-half as fast as poly A. The products of hydrolysis of these polymers were identified as 5′-mononucleotides by their absorption spectra, their chromatographic behavior in Solvents 1 and 3 and their cleavage by purified bull semen 5′-nucleotidase[21]. Other polymers are hydrolyzed to 5′-mononucleotides at rates that are less than 10 % of that observed with poly A. These include poly C, poly AGUC, yeast RNA and RNAs derived from turnip yellow mosaic virus and *A. agilis*. The reason for these low rates is not known, and may, perhaps, be related to degree of polymerization or secondary structure of the polymers.

The hydrolysis of the oligonucleotides, pApA, pApApA, pApApApA and pApApApApA was followed by chromatography in Solvents 1 and 2. The ultimate product was determined to be 5′-AMP by its spectrum, R_F in Solvents 1 and 2, electrophoretic mobility and by the fact that it was hydrolyzed by 5′-nucleotidase[21]. Smaller oligonucleotides accumulate as transient intermediates. Thus, during the hydrolysis of pApApApA to 5′-AMP one can observe the appearance of pApApA and pApA*. Table IV shows the rate of formation of 5′-AMP from these compounds, compared with that from poly A, all measured at saturating concentrations of substrate. Substrate saturation is achieved with $3.5 \cdot 10^{-4}\,M$ pApApApA, $4.5 \cdot 10^{-4}\,M$ pApApA,

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* $R_{3'\text{-AMP}}$, which is the ratio of the R_F of a given compound to that of 3′-AMP has the following values in Solvent 1: pApA, 0.53; pApApA, 0.31; pApApApA, 0.18; pApApApApA, 0.09. In Solvent 4 the values are: pApA, 0.40; pApApA, 0.17; pApApApA, 0.06; pApApApApA, 0.03.

TABLE IV

RATE OF FORMATION OF 5′-AMP FROM POLY A AND FROM CERTAIN OLIGONUCLEOTIDES

The reaction mixture (0.06 ml) contained 0.15 μmole of $MgCl_2$, 2.5 μmoles of Tris, pH 8.2, 0.5 μmole of glutathione, 0.005 μmole of EDTA, substrate and 10 μg of protamine eluate fraction (Table I; specific activity, 15). The mixtures were incubated at 37° and chromatographed in Solvent 1. Regions of the paper containing the 5′-AMP were located in u.v. light and the nucleotide was quantitatively eluted with 0.01 N HCl. The O.D. was measured at 257 mμ. The time of incubation was 0.5 h for poly A and 5.5 h for the oligonucleotides.

Substrate	*Concentration (M)*	*5′-AMP formed μmoles/mg enzyme/h*
Poly A	$6.7 \cdot 10^{-3}$*	15.0
pApA	$2.4 \cdot 10^{-3}$	0.8
pApApA	$1.5 \cdot 10^{-3}$	0.9
pApApApA	$1.4 \cdot 10^{-3}$	1.0

* Expressed as adenine equivalents. All other concentrations are expressed as molar concentration of the given compound. All concentrations were well above the level required to saturate the enzyme.

and $7 \cdot 10^{-4}$ *M* pApA. It is evident from Table IV that 5′-AMP is formed at least 15 times as fast from poly A as from the oligonucleotides*. The pyrimidine derivatives pUpU and pUpUpU are split nearly twice as rapidly as the corresponding adenine oligonucleotides. Mononucleotides with a 2′,3′-cyclic phosphoryl group such as adenosine 2′,3′-phosphate, are not attacked.

Purified protamine eluate fractions as well as fractions from DEAE-cellulose chromatography were tested with the oligodeoxyribonucleotides, d-pTpT, d-pTpTpT and d-pApA. All were studied at a concentration of 10^{-3} *M*; because of insufficient material, substrate saturation curves were not obtained. The rates of formation of mononucleotide from d-pTpTpT and d-pTpT are one-third and one-sixth, respectively, of that found with pApApA, while d-pApA is hydrolyzed about half as fast as d-pTpT.

Specificity requirements of the enzyme

Leukemic cell phosphodiesterase resembles snake venom phosphodiesterase in forming nucleoside 5′-phosphates from polymers and from oligonucleotides bearing a 5′-phosphomonoester end group. This resemblance is borne out in other results. Thus, the compounds ApCp, ApUp and ApApUp, which possess a 3′-phosphomonoester end group are not hydrolyzed. Also, the rates of hydrolysis of ApA and ApApA, two compounds without end groups are very slow. Similar findings have been reported for the venom enzyme[30, 33]. An important difference between the two enzymes was discovered, however, when it was noted that various simple esters of nucleotides, such as benzyl adenosine 5′-phosphate and *p*-nitrophenyl uridine 5′-phosphate are not attacked by the tumor enzyme. Hydrolytic activity against such esters can be detected in crude extracts, but Table V shows that this is almost completely removed as a result of purification. The fractionation produces at least a 75-fold change in the ratio of enzymic activity against poly A to that against *p*-nitrophenyl uridine 5′-phosphate, and the purified fractions hydrolyze several esters at rates that are less than 1/240 of that observed for poly A (Table V).

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* It requires more poly A than oligonucleotides to give substrate saturation, based on adenine equivalents. The data in Table III are consistent with competitive inhibition, by pApA, of the splitting of poly A.

From the data presented here, it appears that a substrate for leukemic cell phosphodiesterase must contain at least two nucleoside residues.

TABLE V

REMOVAL OF HYDROLYTIC ACTIVITY AGAINST CERTAIN *p*-NITROPHENYL ESTERS IN THE COURSE OF PURIFICATION OF LEUKEMIC CELL PHOSPHODIESTERASE

Assays for rates of hydrolysis of poly A and of the *p*-nitrophenyl esters are given under METHODS. Units of activity are expressed as μmoles of product formed per hour at 37°. Comparable results were obtained in another experiment with other, similarly purified, enzyme fractions.

Fraction	*Specific activity (U/mg) against*				*Ratio of specific activities*	
	Poly A	p-*nitrophenyl uridine 5′-phosphate*	p-*nitrophenyl thymidine 5′-phosphate*	p-*nitrophenyl thymidine 3′-phosphate*	$\frac{\textit{Poly A}}{\textit{uridine 5'-ester}}$	$\frac{\textit{Poly A}}{\textit{thymidine 5'-ester}}$
Crude extract	2.42	0.74	1.0	0.2	3.27	2.4
Protamine eluate	16.1	<0.06*	<0.06*	<0.06	>244	>244
DEAE eluate	15.5	<0.07			>222	

* For these measurements the amount of enzyme used was 10 times that required to demonstrate hydrolysis of poly A, and the incubation time was twice as long. Under these conditions there was no detectable reaction with these esters.

Mechanism of the reaction

The cleavage reaction is hydrolytic. There is no absolute requirement for inorganic phosphate. Furthermore, no ADP or ATP can be detected during the splitting of poly A or of adenine-containing oligonucleotides. Control experiments indicate that even small amounts of these compounds are stable upon incubation with the enzyme.

The cleavage of poly A is apparently stepwise, with successive removal of mononucleotide units from an end of the chain, as has been observed for venom phosphodiesterase[31, 32]. This conclusion derives from the fact that pApApApApA and smaller oligonucleotides were never detected at any stage of the hydrolysis of poly A. Random splitting throughout the polynucleotide chain would have given rise to such compounds. Further, if formed, they should have accumulated in the reaction mixture because they liberate 5′-AMP at only one-fifteenth of the rate observed with poly A (Table IV).

RAZZELL AND KHORANA[31] have found that oligodeoxyribonucleotides are degraded by snake venom phosphodiesterase in a stepwise manner, starting from the end of the oligonucleotide chain bearing a 3′-hydroxyl group. HILMOE[32] has confirmed this observation, using polyribonucleotides. In view of this work, an effort was made to discover from which end of the molecule the attack by leukemia phosphodiesterase begins. For this purpose the hydrolysis of pApApApU and pApApApG was investigated, and the reaction products were examined at various time intervals. Unfortunately, pApApA formed as an intermediate was hydrolyzed very much faster than the original tetranucleotides. Apparently (see Table IV) the presence of a terminal uridylic or guanylic acid residue results in a slower rate of attack by the enzyme. As a consequence no conclusions concerning the initial site of hydrolysis could be drawn from serial chromatograms of the reaction mixtures.

DISCUSSION

Leukemic cell phosphodiesterase resembles snake venom phosphodiesterase in its specificity except that various simple esters of nucleoside 5′-phosphates are not hydrolyzed. By contrast, the venom enzyme splits *p*-nitrophenyl thymidine 5′-phosphate more rapidly than any other substrate tested[33]. The tumor phosphodiesterase appears to be the first example of an enzyme hydrolyzing polynucleotides to the mononucleotide stage that requires a nucleoside residue to be present on each side of the susceptible phosphodiester bond. Simple esters in which phosphate is doubly esterified to a single nucleoside residue and to an alkyl or aryl group cannot be cleaved by this new kind of hydrolase. It may be mentioned in this connection that various nucleases such as pancreatic ribonuclease[15], and ryegrass ribonuclease[31] hydrolyze certain simple esters of nucleosides as well as polynucleotides. The same is true for spleen phosphodiesterase[2].

Razzell and Khorana (personal communication) have investigated the cleavage of the *p*-nitrophenyl esters of thymidine 5′-phosphate and thymidine 3′-phosphate. Both types of activity are widely distributed in animal tissues and also occur in bacteria. Enzymes comparable to leukemic cell phosphodiesterase may also be widely distributed, but they would be difficult to find for lack of an easy, specific assay. In most tissues the activity is largely obscured by high concentrations of other, interfering phosphodiesterases and nucleases. Another difficulty is caused by the presence of phosphomonoesterases; most tumor tissues, however, have comparatively low phosphatase activity[35]. Dr. M. F. Singer (personal communications) has found that fractionation of lysates of mouse Ehrlich ascites cells resulted in a 10-fold increase in the ratio of activity against poly A to that against *p*-nitrophenyl uridine 5′-phosphate. This indicates the likely presence in another source of an enzyme that hydrolyzes polynucleotides but not simple esters.

Finally, it may be mentioned that we have demonstrated the formation of nucleoside 5′-phosphates from poly A, poly U, poly AU, pApA, pUpU, etc., in extracts of various other tumor tissues including: (a) an ascitic lymphocytic leukemia L1210[36], (b) an ascitic plasma cell neoplasm, 70429[37], (c) an ascitic mast cell tumor, P815[38], (d) a solid plasma cell tumor, X-5563[39]. These were obtained from Drs. Lloyd Law and M. Potter of the National Cancer Institute. Similar activity was found in extracts of cultured HeLa cells obtained from Drs. N. Salzman and H. Eagle of the National Institute of Allergy and Infectious Diseases. The reactions observed are probably hydrolytic, but fractionation would be required to rule out a phosphorolytic mechanism.

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IN VITRO LIPID SYNTHESIS IN FOWL BLOOD

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SUMMARY

1. Fowl whole blood has been shown to incorporate labelled acetate into both cellular and plasma lipids.

2. Long chain saturated and unsaturated acids are shown to be synthesized and then incorporated into triglycerides, phospholipids and cholesterol esters.

3. "Sterol"-like unsaponifiable substances are also synthesized from acetate.

4. Comparison of the synthetic ability of leucocytes, young erythrocytes and old erythrocytes show that all cells contribute to the synthesis, the most active being the leucocyte and young erythrocyte preparations.

INTRODUCTION

Previous papers have described lipid synthesis in human blood[1], the exchange of these lipids between cells and lipoproteins[2] and the relative contributions to this lipid synthesis of the different cell types[3]. The latter work demonstrated that in human blood the leucocytes carry out the major part of the synthesis.

As a corollary to these studies with the non-nucleated human erythrocyte we have turned our attention to the nucleated fowl cell. Results are presented in this paper on the *in vitro* incorporation of labelled acetate into lipids in both whole blood and fractions enriched in old erythrocytes, young erythrocytes and leucocytes.

MATERIALS AND METHODS

All equipment used up to extraction of the lipid was sterile and siliconized. Blood was drawn into acid–citrate–dextrose and either used directly for the incubation as described below or centrifuged. The plasma was removed and the cells resuspended in a dense medium prepared by dissolving 27 % (w/v) frozen-dried fowl plasma in distilled water, followed by dialysis against several changes of 0.8% NaCl in the cold. Centrifugation of the suspension at 1000 × *g* for 10 min at 4° produced an upper layer rich in leucocytes, which was removed (fraction L), and a lower layer containing very few leucocytes. The erythrocyte layer was subdivided into an upper fraction (Y.E.) and a lower fraction (O.E.). Experiments on the ratio between radioactivity and haemoglobin on similarly prepared fractions after ^{59}Fe administration had shown that the upper fraction (Y.E.) contained a relatively high proportion of young cells. The cells in all fractions were resuspended in the original plasma. Total cell counts and differential counts were made on all fractions using in each case samples of 1000 cells.

To the samples were added 1 μC/ml Na-[Me-^{14}C]acetate, streptomycin-penicillin (2:1 by weight, 2 mg/10 ml blood) and sodium ethylene diamine-tetraacetate (0.1 ml of 0.1 % solution/10 ml blood). After equilibration with 5 % CO_2, incubation was carried out in large stoppered roller tubes at 37° for 6 h, after which the preparations were centrifuged. Plasma samples were removed, cells washed once in a large volume of saline, and both plasma and cell samples were extracted with 20 volumes of ethanol–ether for 12 h at room temperature.

The ethanol–ether solutions were then filtered and evaporated to dryness *in vacuo*. In some experiments the lipid residue was extracted twice with acetone; the insoluble material is referred to as phospholipid and the soluble portion as neutral fat. This neutral fat fraction was then further fractionated by chromatography using a liquid-solid system as described previously[1]. The methods of saponification, methylation and separation by gas liquid chromatography have been published earlier[1].

RESULTS

As was demonstrated with human blood[1] both cellular and plasma lipids were found to be extensively labelled in two experiments after incubating whole fowl blood with radioactive acetate (Table I).

Separation of the fatty acids from these fractions and determination of specific activities showed the pattern of labelling to be similar to that in human blood (Table II). Both the saturated acids up to C_{18} and the monoenoic, dienoic and trienoic C_{18} and the trienoic and tetraenoic C_{20} acids were labelled, indicating considerable biosynthesis. The fatty acid composition of these lipids is given in Table III.

In another experiment the lipid classes from the cells and the plasma were separated by chromatography on silica gel. The specific activities of the fatty acids

TABLE I

SPECIFIC ACTIVITY OF NEUTRAL FAT AND PHOSPHOLIPID AFTER INCUBATION OF WHOLE FOWL BLOOD WITH [Me-^{14}C]ACETATE

Expt.	*Neutral fat*		*Phospholipid*	
	Mixed cells	*Plasma*	*Mixed cells*	*Plasma*
I	940*	418	876	1975
II	1417	783	550	837

* Counts/min/mg.

TABLE II

SPECIFIC ACTIVITIES OF INDIVIDUAL FATTY ACIDS FROM NEUTRAL FAT AND PHOSPHOLIPID OF COMBINED RED AND WHITE CELLS, AND PLASMA

	Neutral fat		*Phospholipid*	
	Cells	*Plasma*	*Cells*	*Plasma*
Myristic	N.D.*	1253**	N.D.	N.D.
Palmitic	556	535	240	143
Stearic	297	311	132	45
Oleic	125	9.5	35	50
Linoleic + linolenic	23.4	8.4	14	30
Arachidonic	N.D.	127	72	44
C_{20} triene	N.D.	N.D.	286	278

* N.D., not determined.
** Counts/min/mg.

TABLE III

FATTY ACID COMPOSITION* OF COMBINED RED AND WHITE CELLS AND PLASMA, NEUTRAL FAT AND PHOSPHOLIPID (FROM TABLE II)

	Neutral fat		*Phospholipid*	
	Mixed cells	*Plasma*	*Mixed cells*	*Plasma*
Myristic	trace	0.6	trace	trace
Palmitic	20.6	20.7	19.3	23.7
Stearic	21.2	9.7	18.8	23.4
Oleic	18.7	31.2	13.9	16.7
Linoleic	38.3	30.6	37.5	22.8
Arachidonic	N.D.**	4.2	5.6	8.7
C_{20} triene	N.D.	trace	1.5	4.0

* Major components only.
** N.D., not determined.

and non-saponifiable substances in each fraction were determined after saponification and the results are given in Table IV. Unlike human blood the fatty acids and non-saponifiable material of the cholesterol esters were labelled. Maximum labelling was found (as in human blood) in the triglyceride fraction. The mixed fraction of sterol and diglyceride contained a large amount of labelled sterol (97 % of the fraction). In general fowl blood resembles human blood in its ability to synthesize long chain saturated and unsaturated acids from acetate, to incorporate these acids into phospholipids and triglycerides and to secrete these complex lipids into the plasma. The main difference between the two systems lies in the ability of the fowl red and white cells to incorporate fatty acids into cholesterol esters and also to synthesize more unsaponifiable "sterol"-like substances.

Relative contribution of the different cell types

The results of incubating labelled acetate with fractions enriched in leucocytes, young erythrocytes and old erythrocytes are given in Table V. It is evident that the highest incorporation of acetate into lipid has taken place in the leucocyte-rich fraction. Thus in whole fowl blood, as in human blood, leucocytes must make a substantial contribution to total lipid synthesis. However, the rate of incorporation of label into both unsaponifiable lipid and fatty acids in the erythrocyte-rich fractions, even after correction for the leucocyte count in the red cell preparation, is still large, so that the substantial proportion of the synthesis observed must be due to erythrocytes.

An indication of the relative rates of synthesis by the several-cell types can be obtained by calculating the incorporation in each fraction due to each of the cell types present and dividing the result by the corresponding cell count. The figures in Table VI summarize the results of such a calculation. They suggest that on average leucocytes synthesize both fatty acids and non-saponifiable substances at least 300 times as fast as young erythrocytes and they in turn synthesize these substances at least 3 times as fast as older erythrocytes. Another experiment gave similar figures for various cell fractions and relative rates of synthesis.

Specific activities of the long chain fatty acids isolated from the total lipids of the leucocyte-rich, young erythrocyte-rich and old erythrocyte-rich fractions are given in Table VII. The activity of most of the individual acids increases in the order: old erythrocyte, young erythrocyte, leucocyte, in agreement with the specific activity of the total lipid.

DISCUSSION

The experiments quoted suggest that nucleated fowl erythrocytes—in contrast to non-nucleated human erythrocytes—retain some capacity for synthesis of both unsaponifiable lipid and fatty acids. The rate of synthesis by erythrocytes is, however, much less than that by leucocytes. The capacity of erythrocytes for synthesizing lipid is paralleled by their ability to incorporate labelled glycine into haemoglobin. This synthesis takes place throughout the life-span of nucleated avian erythrocytes[4], but not in mature non-nucleated erythrocytes[5]. In view of the known part played by acetyl-coenzyme A in lipid synthesis, it may be relevant that in avian erythrocytes,

TABLE IV

SPECIFIC ACTIVITIES OF TOTAL FATTY ACID AND NON-SAPONIFIABLE MATERIALS IN LIPID COMPONENTS OF COMBINED RED AND WHITE CELLS AND PLASMA

Specific activities from combined extracts of Expts. 1, 2.

	Hydrocarbons + cholesterol esters		*Largely triglycerides*		*Sterol + diglycerides*		*Unknown eluted with ether*		*Unknown eluted with acetone*	
	Mixed cells	*Plasma*	*Mixed cells*	*Plasma*	*Mixed cells*	*Plasma*	*Mixed cells*	*Plasma*	*Mixed cells*	*Plasma*
Acids	594	1.9	3490	1970	109	520	1115	474	227	186
Non-saponifiable substances	427	3.8	186	29	146	210	268	58	16	12.6
Percentage non-saponifiable substances in fraction	22.6	80	15	6.8	96	98	49	30	25	25

TABLE V

CELL POPULATION AND LIPID LABELLING

Fraction	*Erythrocyte count*	*Leucocyte count*	*Fatty acid specific activity (counts/min/mg)*	*Fatty acid product of specific activity and weight in mg total activity (counts/min)*	*Unsaponifiable lipid specific activity (counts/min/mg)*	*Unsaponifiable lipid product of specific activity and weight in mg lipid total activity (counts/min)*
L. cells	$0.25 \cdot 10^6$	$12.8 \cdot 10^3$	830	79,650	510	1221
L. plasma	$0.25 \cdot 10^6$	$12.8 \cdot 10^3$	729	56,880	351	596
Y.E. cells	$2.28 \cdot 10^6$	$0.9 \cdot 10^3$	632	41,020	502	1208
Y.E. plasma	$2.28 \cdot 10^6$	$0.9 \cdot 10^3$	370	44,400	300	300
O.E. cells	$4.37 \cdot 10^6$	$0.2 \cdot 10^3$	364	10,680	155	631
O.E. plasma	$4.37 \cdot 10^6$	$0.2 \cdot 10^3$	592	34,400	225	534

TABLE VI

RELATIVE SYNTHETIC ACTIVITY OF DIFFERENT CELL TYPES AFTER CORRECTION FOR MIXED CELL POPULATION

Cell type	*Counts in fatty acid/min/cell*	*Relative synthetic activity for fatty acids*	*Counts in non-saponifiable materials/min/cell*	*Relative synthetic activity for non-saponifiable substances*
Leucocyte	10.5	1000	0.14	560
Young erythrocyte	$33.2 \cdot 10^{-3}$	3.4	$0.5 \cdot 10^{-3}$	2.0
Old erythrocyte	$9.8 \cdot 10^{-3}$	1.0	$0.25 \cdot 10^{-3}$	1.0

TABLE VII

SPECIFIC ACTIVITIES OF FATTY ACIDS AND METHYL ESTERS ISOLATED FROM DIFFERENT CELL TYPES

Activity in counts/min/mg.

Acid	*Leucocytes*	*Young erythrocytes*	*Old erythrocytes*
nC_{16}	1420	1000	81
nC_{18}	900	926	159
Oleic	120	70	9.0
Linoleic	34	40	15.0
Arachidonic	54	50	162
C_{20} triene	86	30	N.D.

unlike mammalian erythrocytes, there is evidence of the presence of a tricarboxylic acid cycle[6].

Because the cell population in whole blood is roughly 1 leucocyte to 300 young erythrocytes and 1000 mature erythrocytes, each cell type contributes approximately equally to the total lipid synthesis in whole blood.

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METABOLISM AND FUNCTIONS OF PHOSPHATIDES

SPECIFIC FATTY ACID COMPOSITION OF THE RED BLOOD CELL MEMBRANES

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SUMMARY

In attempting to establish a specific lipid composition of biological interfaces, the fatty acid composition of the lipid moiety of the red cell membrane of a number of mammals was determined by means of gas–liquid chromatography. A rather constant and characteristic fatty acid pattern proved to occur in the membranes of the red cells of each species. Quantitatively striking differences were found between the fatty acid compositions of the red cell membranes of the various mammals. Most pronounced were differences in the palmitic acid and oleic acid content. A gradual decrease in the ratio palmitic acid–oleic acid was shown in the red cell membranes of: rat, man, rabbit, pig, horse, ox and sheep in that order. Since this arrangement rather resembled the sequence based on permeability properties of these membranes taken from the literature, a possible relationship between fatty acid composition and permeability of membranes is discussed.

INTRODUCTION

The obvious differences in permeability of the numerous interfaces which regulate the transport between cells and their environment, and also between the cell particles inside the cell, suggest a specific frame of each of these various biological membranes. Theories attempting to formulate the arrangement of molecules in the biological membranes, all recognize the importance of complexes formed by an association of proteins with lipids[1-4]. Divergencies in the properties of the interfaces may be dependent on the orientation, the types, and the ratio of the molecules or molecular fragments, participating in the various chemical and physico-chemical combinations. Whereas the variability of the protein moiety of the corresponding lipoproteins appears theoretically to be nearly unlimited, the variation of the lipid part, consisting mainly of cholesterol and phosphatides, seems to be more restricted. Differences in the fatty acids of the lipid molecules, however, may also contribute to a distinction between the various biomembranes. The chain length and the degree of saturation of the fatty acid constituents may play a part in the functional mechanism of the

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[*] Deceased June 6th, 1959.

biological interfaces. Hence the question arose, whether different biomembranes are characterized by a specific fatty acid composition.

A difficulty in studying membrane lipids is to isolate them specifically from the tissues, without contamination with other lipids. Of course this difficulty disappears if one can isolate the membranes of cells or cell particles. This seems to be possible with mammalian red cells, where, by osmotic means, posthemolytic residues ("ghosts") may be obtained that should represent the plasma membrane as closely as possible. It was therefore decided to make comparative analyses of the fatty acid composition of ghost lipids of red cells obtained from rat, rabbit, pig, sheep, ox, horse, and of man.

EXPERIMENTAL

Preparation of ghosts

Erythrocytes were gained by centrifugation of whole blood, previously defibrinated by shaking with glass beads. The cells were washed 3 times with 0.9 % NaCl solution at room temperature. After hemolysis for 18 h in 10 volumes of water saturated with CO_2, at 3°, ghosts were prepared according to the CO_2 method of PARPART[5], omitting, however, the saline washings which were suspected to give a loss of wanted material. The yield of posthemolytic residues recovered from the cells of different species after lyophilization varied from about 3 to 5 g/l of whole blood.

Extraction of the lipids

Lipids were extracted by treating about 4 g of dry ghosts with 500 ml of ethanol–ether (3:1; v/v) at room temperature for 18 h. The extraction was repeated twice in a similar way, but now for 1 h. The combined extracts were evaporated *in vacuo* under nitrogen, leaving 0.8–1.2 g of membrane lipids. These lipids were stored *in vacuo* in the dark at 2°.

Saponification

Saponification was carried out by refluxing about 250 mg of the extracted lipids with 10 ml of a N NaOH solution in ethanol in a nitrogen atmosphere for 2 h. After diluting with water unsaponifiable material was removed by extraction with ether, freed from peroxides as usual. The resulting aqueous soap solution was acidified with sulfuric acid and the fatty acids were extracted with ether.

Esterifying

After drying the etheric solution with anhydrous Na_2SO_4 the fatty acids were esterified with a diazomethane solution in ether. The solution of fatty acid methyl esters was filtered and evaporated *in vacuo*.

Gas–liquid chromatography

The fatty acid methyl esters, diluted some 5 times with ether, were analysed by means of gas chromatography. A Perkin-Elmer 154 B Vapor Fractometer was used, supplied with an electronic temperature regulator (as in Perkin-Elmer 154 C) so as to obtain a good base line at high temperatures. Condensation of products before they reached the column caused a very bad tailing on the chromatograms. This was remedied by additional heatings both of the injection block and of the tube connecting the injection block with the column. Also the venting tube had to be warmed up electrically to prevent condensation of the vapours as they left the column. As the thermistor katharometer has no high sensitivity at the used high temperatures, and as large samples had a bad effect on the separation especially on column b (see below), the sensitivity of the used 5 mV Leeds and Northrup Speedomax recorder was raised 3.5 times. Furthermore, this recorder was modified so as to come to a complementary chart speed, one sixth of the normal chart speed of 1 cm/min.

Two different columns were used:

Column a: Silicon grease stationary phase. Dow Corning high vacuum silicon grease was purified by solving it in ethyl acetate and precipitating with ethanol[6]. The solid support was an acid washed, coarse fraction of Celite 545 (*cf.* ref. 7). The inner diameter of the 2-m stainless steel column was 4½ mm. The working temperature was 225° and the flow 50 ml of He/min.

Column b: Diethylene glycol succinate stationary phase. Poly diethylene glycol succinate solution was obtained from Research Specialties Co. The solvent was evaporated and any low boiling material was eliminated by heating to 200° *in vacuo*. The solid support consisted of 80–100 mesh Celite, washed with acid and alkali as described by LIPSKY *et al.*[8]. Use was made of two one meter columns of pyrex glass with an inner diameter of 4½ mm, in series. The working temperature was 200°, the flow 105 ml of He/min.

In both cases the inlet pressure was 30 lbs/in^2, the outlet atmospheric. The stationary phases (1 part) were solved for column a in ether and for column b in acetone and mixed with their respective solid supports (3 parts). The solvent was evaporated *in vacuo* with frequent shaking and heating up to 100°.

With column a the methyl arachidate peak appears within 75 min, with column b within 15 min. As a rule for column a chromatograms the recorder was used at normal sensitivity and low chart speed, for column b chromatograms at high sensitivity and high chart speed. The separation by column b was affected much more by too large sample volumes than the separation by column a*.

Samples of 5–50 µl of the methyl ester solutions in ether were put onto the column by means of a 250-µl hypodermic syringe.

RESULTS

Qualitatively some, or all, of the following fatty acid constituents are found in the lipid samples from red cell membranes: lauric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic and arachidonic acids. No arachidic acid was found. Identification was carried out by comparing the retention times on the two different columns with the retention times of the members of known mixtures of fatty acid methyl esters. This identification was enhanced by the fact that with column a the saturated esters appear next to the unsaturated ones with the same chain length, the latter without adequate separation from one another, whereas with column b the saturated esters appear first and the unsaturated ones are very well separated. Moreover, hydrogenation of the fatty acid methyl esters with the aid of Pt catalyst and subsequent rechromatographing gave additional information. Another confirmation of our identifications was obtained by reversed phase paper chromatography of the free fatty acids*.

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* Also other columns were tried with the following stationary phases: laboratory-made poly diethylene glycol succinate, poly diethylene glycol adipate (LAC-1-R 296, Cambridge Inds. Co., and Reoplex 400, Geigy, respectively) and Apiezon N, and furthermore the columns A, B and P as obtained from Perkin-Elmer Co. However, the described combination of columns a and b showed to be the most satisfactory. Moreover, column a was chosen since it was nearly undestroyable: after two years of use there is no appreciable loss of separation, and retention times are only slightly diminished. Furthermore, column b gave the most rapid analysis of our esters, and the separation was not even less than with column P of Perkin-Elmer Co.

Quantitative determinations of the fatty acid composition of our lipid samples were carried out by measuring peak areas on the gas chromatograms. For this purpose mainly chromatograms with column b were used because of the better separation of all components of our samples on this column. Areas were determined by multiplying the peak height by the width at half height. Analysis of weighed amounts of standard mixtures of fatty acid methyl esters showed peak areas to be directly proportional to component weights with the same proportionality factor for each ester from laurate to arachidate, so peak area percentage of total measured peak areas is identical with weight percentage of fatty acid methyl ester in the sample.

In Fig. 1 some representative gas chromatograms are shown. The quantitative results of all of our experiments are summarized in Table I.

In the second column of this table the number of animals is given from which blood was pooled before the analysis. The figures in the following columns represent

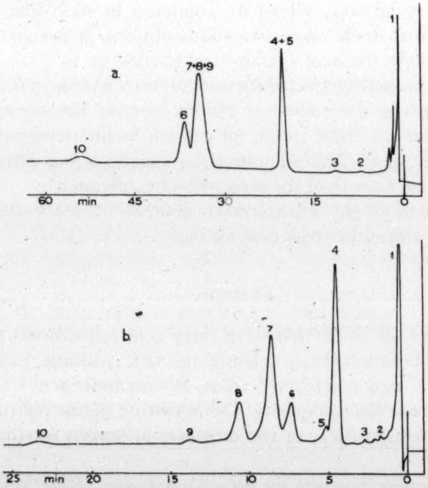

Fig. 1. Examples of actual gas chromatograms. a, fatty acid methyl esters from pig ghost lipids on column a; b, same on column b. For details see text. 1, ether; 2, laurate; 3, myristate; 4, palmitate; 5, palmitoleate; 6, stearate; 7, oleate; 8, linoleate; 9, linolenate; 10, arachidonate.

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* Whatman No. 1 paper was impregnated with petroleum, according to WAGNER *et al.*[9]. The fatty acids were stained by the Cu dithiooxamide method, and iodine vapour revealed only the unsaturated ones.

TABLE I

PERCENTAGES OF FATTY ACIDS IN GHOST LIPIDS

Percentages are given of total measured peak areas on gas chromatograms of column b of the methyl esters from laurate to linolenate. + denotes that the peak is too small for accurate measurement. The standard errors of the means* are placed between brackets.

Animal		*Number of animals*	*Laurate*	*Myristate*	*Palmitate*	*Palmitoleate*	*Stearate*	*Oleate*	*Linoleate*	*Linolenate*
Rat		17	+	+	45	—	21.5	17.5	15.5	0.5
Rat		8	+	1	41	2	23.5	18.5	14	—
	Mean		+	0.5 (0.5)	44 (1.9)	0.5 (1.0)	22 (1.0)	18 (0.5)	15 (0.7)	+
Man (A, rh. pos.)		1	+	+	40.5	2.5	15	25	16	1
Man (A, rh. pos.)		3	+	—	38.5	2.5	15	27	17	+
Man (B, rh. pos.)		1	+	+	37.5	2	13	25.5	20	2
Man (B, rh. neg.)		1	—	+	37	—	14.5	25.5	20	3
Man (O, rh. pos.)		2	+	+	38.5	—	16.5	29	14	2
Man (O, rh. pos.)		2	+	+	34	+	16.5	28.5	19	2
Man (O, rh. pos.)		3	—	+	36	2	15	25.5	18	3.5
Man (O, rh. pos.)		1	—	+	39.5	2	17.5	26.5	13.5	1
	Mean		+	+	37.5 (0.7)	1.5 (0.4)	15.5 (0.5)	26.5 (0.6)	17 (0.8)	2 (0.5)
Rabbit		5	—	—	40	5	10.5	28.5	16	—
Rabbit		4	1	0.5	33	4.5	11.5	24	23.5	2
Rabbit		7	+	+	34	3	11	23	28	1
	Mean		+	+	36 (2.1)	4 (0.7)	11 (0.3)	25 (1.6)	23 (3.7)	1 (0.6)
Pig		1	—	+	29.5	2.5	10	38	20	—
Pig		1	+	0.5	31	3	17	31	14	3.5
Pig		3	+	+	32.5	1.5	14	36.5	14.5	1
Pig		2	+	+	26	1.5	16	33.5	22	1
	Mean		+	+	30 (1.6)	2 (0.4)	14.5 (1.3)	35 (1.3)	17.5 (2.0)	1 (0.6)
Horse		1	+	+	25.5	4	19	30.5	19.5	1.5
Horse		2	+	+	25.5	4	18.5	34	18	—
Horse		3	+	+	18.5	1	11.5	26	40.5	2.5
Horse		2	+	+	19	2.5	12.5	32.5	30	3.5
	Mean		+	+	21.5 (1.9)	2.5 (0.8)	14.5 (1.9)	30 (2.0)	29.5 (5.5)	2 (0.8)
Ox		1	—	+	21.5	3.5	17	47	10.5	+
Ox		3	—	+	11	3	14	53	15.5	3.5
Ox		2	—	+	12	4.5	14	52	16	1.5
	Mean		—	+	13 (2.7)	3.5 (0.5)	14.5 (0.8)	52 (1.6)	15 (1.4)	2 (1.0)
Sheep		1	1.5	1	17	3.5	10	55	10	2.5
Sheep		3	—	+	10	3.5	8	63.5	8.5	7
Sheep		3	+	—	13.5	2	6	62	11	5.5
	Mean		+	+	12.5 (1.7)	3 (0.6)	7.5 (1.0)	61.5 (2.0)	10 (0.9)	5.5 (1.1)

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* The mean values have been calculated taking into account the number of animals of each experiment. This procedure was justified by the fact that the standard deviation of analyses of one sample of blood which was done in quadruplicate, was significantly lower than the standard deviation of experiments of different samples of blood.

weight percentages of the methyl esters found. Data on the amount of arachidonic acid are not given, for not all of our chromatograms were continued until arachidonic acid methyl ester left the column. However, some of the samples studied, mainly of the animals first mentioned in the table, contained considerable amounts of this acid. Fatty acids of higher unsaturation or with a longer carbon chain were neglected.

Examination of the table indicates that in all studied species the fatty acid composition of the erythrocyte membrane reveals for a certain species a strikingly characteristic and constant pattern. Although in most cases it was not possible to establish the origins and antecedents of the animals, no marked differences were noticed in the analyses carried out in various seasons, with involved differences in the diets of the animals. Although we worked up our samples under nitrogen, incidentally some oxydation may have taken place of linoleic and linolenic acids. Probably this does not explain the variability in the amount of linoleic acid obtained in the analyses of horse ghost lipids. However, we know that only the horses of the third experiment, giving the highest amount of linoleate, were grown up in East Europe.

Moreover, not only a rather constant fatty acid composition of the erythrocyte membrane of one species appears to exist, but pronounced differences are also noted in this respect between the various species. The red cell membranes of the series rat, man, rabbit, pig, horse, ox and sheep show a gradual decrease of the palmitic acid concentration. A corresponding shift in the oleic acid amount—but in opposite direction—proved to occur in the same order, with a low percentage of oleic acid in the red cell membrane of the rat, up to an extremely high amount in the sheep.

DISCUSSION

The results of our experiments indicate a specific fatty acid composition of the lipid moiety of the red cell membrane, which is qualitatively almost the same, but quantitatively strikingly different for the various species in question. The notable differences found in the fatty acid patterns of the various red cell membranes are remarkable, since the general function of the red cells in mammals will be the same, so these differences prompt the question whether there is any relationship between the specific lipid composition and the permeability properties, which are known to vary widely.

Among the frequent literature references on the possible relations between chemical composition of red cell surfaces and their permeabilities, it was HÖBER[10] who assumed on the grounds of the data available in 1935 that the area of the cell surfaces occupied by lipid, must differ from species to species, to explain their differences in permeability to lipid soluble compounds. PARPART AND DZIEMIAN[11] suggested in 1940 that the permeability should be dependent on the ratio of different types of lipids and on the amount of lipoprotein complexes. In a more recent paper PARPART AND BALLENTINE[4] remarked that no major differences in the proportions of lipid types have been found in the red cells of various species, nor has any significant correlation between lipid content and the large permeability differences between the red cells of various vertebrates been observed. A comparison of our gas-chromatographic data on the fatty acid composition with figures on the permeability properties of the membranes as given in the literature, discloses that such a relationship might exist, though being of another kind than was assumed by these authors.

In Fig. 2 are shown in the first place for each animal the means of the values for the different fatty acids of Table I. From rat *via* man, rabbit, pig, horse and ox to sheep a steady decrease is shown in the amount of palmitic acid, accompanied by an increasing amount of oleic acid in the ghost membrane lipids. In the second place in Fig. 2 pictures are shown on the permeability of the membranes of the same animals as given by JACOBS, GLASSMAN AND PARPART[12], and by HÖBER AND ØRSKOV[13]. They measured the hemolysis times of red cells in isotonic solutions and figured out that there may be a relation between permeability (P) and hemolysis time (T), as given by $P = c/T$, in which c is a constant[12]. In the pictures the hemolysis times in an isotonic solution of urea, thiourea, glycerol and ethylene glycol respectively are plotted on four perpendicular axes in a logarithmic scale. Rather surprising was the fact that by virtue of these permeability pictures, the studied animals could be placed in the same sequence as on the grounds of the fatty acid analyses, showing this time a gradual shift in permeability properties.

Perhaps no less important is the steady increase in quite the same order in the total percentage of unsaturated acids in the membranes, as can also be seen from Fig. 2 (heavy line). This appears to justify the assumption made by BOOIJ AND BUNGENBERG DE JONG[2], that one of the factors influencing the permeability, should be "the number of double bonds in the carbon chain of the lipids".

In Fig. 2 the hemolysis picture of the horse had to be omitted, as the consulted literature did not provide complete data on this animal. In Fig. 3 the possible relation is demonstrated between the permeability to glycerol and the concentration of oleic acid, one of the most conspicuous acids*. Here can be seen that also the hemolysis time of horse erythrocytes in an isotonic solution of glycerol, given by JACOBS *et al.*[14] in an older publication, is in accord with the parallel found between permeability and fatty acid composition of the lipid moiety of the studied membranes.

The facts appear to support, so far, the assumption that the lipid part of the various biological interfaces may contribute to their distinction and specificity. The noticed differences in the fatty acid pattern of the studied homologue membranes need additional research in several directions to fully justify a general conclusion. One problem, which would perhaps at the moment be difficult to dissolve, concerns the possible differences in fatty acids of the various interfaces of different tissues within one species. Within the scope of the specificity problem of the red cell membrane, not only an extension to other classes of vertebrates is desirable, but it appears also valuable to approach the problem indirectly by determining the influence of diet components on the specific fatty acid composition of the membranes. Whereas an augmentation of the proportion of unsaturated fatty acids in the diet is known to alter the component fatty acids of depot fats remarkably, we suppose that such a reflection in those membrane fatty acids having specific functions, must occur to a less extent. Furthermore, it seems interesting to make a study of the red cell membranes of organisms under pathological conditions.

Another way of tackling the problem in discussion may be a determination of the fatty acids of the different types of lipids in the various red cell membranes. At our laboratory work is in progress now to differentiate between fatty acids from neutral lipids, lecithins and other phosphatides by analysing these fractions of ghost membrane lipids one by one.

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* The correlation coefficients (r) have been determined between the hemolysis times for glycerol (a) and glycol (b) and the amounts of palmitate (x) and oleate (y) in the ghost lipids, and were found to be significant.

$r_{ax} = -0.905, p < 0.01$; $r_{ay} = +0.773, p < 0.05$; $r_{bx} = -0.956, p < 0.001$; $r_{by} = +0.868, p = 0.01$

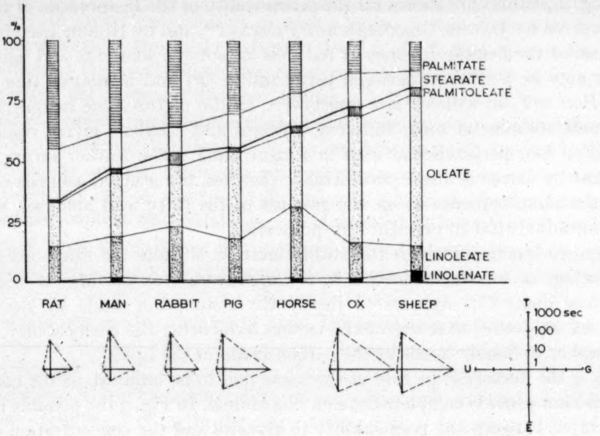

Fig. 2. Comparison of fatty acid percentages of ghost lipids with permeability properties of red cells. Upper part: Means of the fatty acid methyl ester values from Table I, omitting the minor components laurate and myristate. The heavy line separates saturated and unsaturated acids. Lower part: G, hemolysis times in isotonic solutions of glycerol; E, ethylene glycol; U, ureum; and T, thioureum, respectively, on a logarithmic scale as indicated. The picture for the pig is calculated from values of Höber *et al.*[13], the other pictures are taken directly from Jacobs *et al.*[12]. No complete values for the horse are given by these authors.

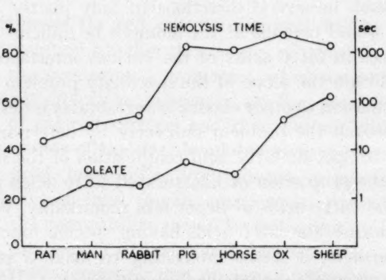

Fig. 3. Comparison of oleic acid content of the ghost lipids with the permeability for glycerol. Lower curve: Mean percentages of oleate from Table I (left hand ordinate). Upper curve: Hemolysis times of erythrocytes in isotonic solution of glycerol[12–14] (right hand ordinate).

We are aware that the results obtained so far, need extensive supplementary evidence to secure more general conclusions on the relation between fatty acid composition and permeability properties of membranes, conclusions which may be fruitful for a further elucidation of structure and mechanism of biological interfaces.

ACKNOWLEDGEMENTS

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QUELQUES REMARQUES COMPLÉMENTAIRES SUR L'HYDROLYSE DES TRIGLYCERIDES PAR LA LIPASE PANCRÉATIQUE

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SUMMARY

Some additional remarks on the hydrolysis of triglycerides by pancreas lipase

1. *In vitro* lipolysis of triglycerides is impeded by difficulties due to the conflict between hydrolysis, on the one hand, and progressive inactivation of the lipase, on the other. The maximum rate of hydrolysis attainable consequently depends in the first place on the relation between the amounts of enzyme and substrate present. It is found that, when the initial proportion of the enzyme is increased, the lipolysis tends to become total.

2. However, even when lipolysis is ultimately total, considerable quantities of transitory partial glycerides are formed while lipolysis is going on.

3. The "activating" effect on lipolysis commonly attributed to calcium ions and bile salts is discussed.

4. Some consequences of these *in vitro* observations on the nature of the products absorbed by intestinal mucosa and on the mechanism of triglyceride resynthesis in this mucosa, are briefly considered.

INTRODUCTION

Des expériences déjà anciennes[1-3] ont montré que: (a) l'hydrolyse *in vitro* des triglycérides par la lipase pancréatique paraît s'arrêter spontanément bien avant que toutes les liaisons ester du substrat aient été coupées; (b) lorsque la vitesse de l'hydrolyse devient nulle, les mélanges engendrés contiennent des quantités considérables de glycérides partiels; (c) ces derniers sont principalement des diglycérides en l'absence d'ions Ca^{++} et des monoglycérides en leur présence.

De tels résultats peuvent être interprétés comme signifiant que la lipolyse *in vitro* est normalement incomplète et que le calcium incite la lipase à convertir les diglycérides en monoglycérides. La première proposition est d'ailleurs en bon accord avec les difficultés notoires qu'éprouve la lipase à hydrolyser les glycérides partiels qu'elle a elle-même formés. Ces difficultés proviennent, on le sait, de l'existence d'hydroxyles dans les molécules en question et de la spécificité de position de la lipase, grâce à laquelle l'enzyme arrache beaucoup plus vite les chaînes externes que les chaînes internes[4-6]. Quant à la deuxième proposition, elle serait susceptible de fournir une explication plausible à l'effet "activateur" communément attribué au calcium pendant la lipolyse.

Toutefois, des expériences ultérieures effectuées avec de la pancréatine de porc[7] et du suc pancréatique de rat[8,9] suggèrent que la lipolyse *in vitro* peut quelquefois aller très loin. Le suc pancréatique de rat en présence de bile paraît même engendrer dès le début beaucoup de glycérol libre[9], ce qui excluerait dans ce cas la formation transitoire de quantités appréciables de glycérides partiels.

Bien que les conditions régnant dans le duodénum pendant la digestion des triglycérides soient vraisemblablement fort différentes de celles de la lipolyse *in vitro*, il est intéressant de déterminer avec précision les facteurs contrôlant le taux maximum d'hydrolyse susceptible d'être atteint au cours de ce dernier phénomène. Les glycérides se présentent en effet devant la paroi intestinale dans l'état où les a mis la lipase. Si l'hydrolyse est complète, ce sont des acides qui pénètrent dans la muqueuse et qui servent à la resynthèse des triglycérides à partir d'un précurseur tricarboné soluble[10]. Si l'hydrolyse est au contraire incomplète, la paroi absorbe un mélange d'acides gras et de glycérides partiels, lesquels peuvent servir directement à la resynthèse au sein d'une phase insoluble.

L'objet du présent travail est de montrer que l'un des facteurs essentiels contrôlant le taux d'hydrolyse est la quantité relative de lipase par rapport aux triglycérides à hydrolyser. Quelques observations concernant le rôle des ions calcium et des sels biliaires pendant la lipolyse sont également discutées.

TECHNIQUES

La plupart des techniques ont déjà été décrites. La lipase est employée, soit à l'état de pancréatine de porc, soit sous la forme de préparations apparemment pures obtenues par précipitation, adsorption et électrophorèse d'extraits de pancréatine[11]. Toutes choses égales d'ailleurs, les résultats obtenus sont identiques dans les deux cas. Le nombre d'unités lipase est déterminé par titrimétrie à pH constant sur une émulsion d'huile d'olive dans la gomme arabique[11,12]. La composition finale des mélanges, c'est à dire leur teneur en triglycérides, diglycérides, monoglycérides, glycérol et acides libres est déterminée par titrage[3] ou chromatographie[13].

Les conditions expérimentales adoptées pour les lipolyses sont les suivantes:

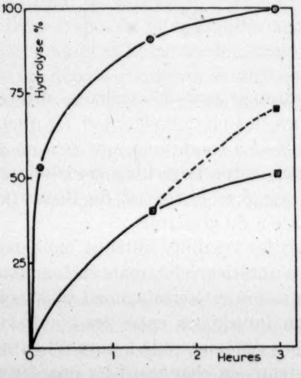
Essais en présence de calcium: Tampon chlorure d'ammonium–ammoniaque 0.02 M pH 8.0 contenant assez de chlorure de calcium pour fournir ½ Ca^{++} par chaîne libérable et, sauf spécification contraire, 0.2 % de taurocholate de Na. Pendant l'expérience, le pH est maintenu à 8.0 par addition d'ammoniaque 5 M.

Essais en l'absence de calcium: Tampon phosphate 0.1 M pH 8.0 contenant, sauf spécification contraire, du taurocholate de Na à la concentration de 0.2 %. Le pH est maintenu à 8.0 en ajoutant avec précaution et sous agitation de la soude 0.5 N. Toutes les expériences sont faites à 37°.

RÉSULTATS

Il est manifeste qu'en insolubilisant les savons solubles formés par la lipolyse, les ions Ca^{++} facilitent beaucoup l'interprétation générale des résultats. Ils diminuent en effet la probabilité de certains phénomènes parasites, tels que la resynthèse, l'échange entre chaînes libres et combinées et l'arrêt prématuré de la réaction dû à l'encombrement de l'interface. Nos premières expériences, reproduites dans les Figs. 1 et 2, ont donc été réalisées en présence d'assez de calcium pour insolubiliser toutes les chaînes susceptibles d'être libérées (3 ions Ca^{++} pour 2 moles de triglycérides).

Les courbes des Figs. 1 et 2 seront commentées un peu plus loin. Notons simplement ici que: (a) Quand les quantités de lipase sont relativement faibles par rapport aux quantités de triglycérides à hydrolyser (13.3 unités/g; Fig. 1), la lipolyse paraît bien, comme on l'avait constaté jusqu'ici, s'arrêter, ou au moins ralentir très fortement, avant que toutes les chaînes aient été libérées. (b) Mais, avec 400 unités/g, le taux d'hydrolyse augmente régulièrement en fonction du temps pour atteindre bientôt 100 %. (c) Le taux maximum susceptible d'être atteint au bout de 3 h (Fig. 2) s'élève quand le rapport lipase/triglycérides s'accroît. Au début, tout accroissement



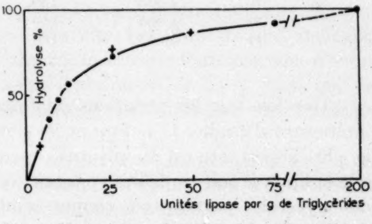


Fig. 2. Variation en fonction du rapport lipase/triglycérides du taux d'hydrolyse au bout de 3 h (expériences avec calcium et taurocholate). On fait varier systématiquement le rapport lipase/triglycérides, soit en augmentant la quantité d'enzyme pour un poids donné de substrat (points noirs), soit en augmentant le poids du substrat pour un nombre donné d'unités enzymatiques (croix).

Fig. 1. Influence de la quantité de lipase sur la cinétique de la lipolyse en présence de calcium et de taurocholate. □ et ⊙: 13.3 et 400 unités lipase/g de triglycérides, respectivement. ·····, cinétique du phénomène quand on ajoute à nouveau 13.3 unités lipase au cours de la 1ère expérience.

de ce rapport exerce un effet considérable. Avec 25 unités/g, le taux est déjà de 70 %. Puis, la courbe s'infléchit et arrive asymptotiquement à l'ordonnée 100 %. (d) Une nouvelle addition de lipase au cours de la 1ère expérience de la Fig. 1 (courbe en pointillé de cette figure) redonne à la lipolyse une vitesse analogue à sa vitesse initiale.

D'autres essais ont ensuite été faits dans le but de mieux connaître l'influence des ions calcium et des sels biliaires sur le déroulement général de la lipolyse. Les résultats de ces essais sont réunis dans le Tableau I. On voit tout de suite que: (a) En 180 min, 400 unités lipase sans calcium donnent un taux d'hydrolyse plus faible que 260 unités avec calcium en 15 min. Les lipolyses sans calcium semblent donc être beaucoup plus malaisées. (b) Néanmoins, quand on augmente considérablement les quantités de lipase (4,000 unités/g) on atteint en 180 min un taux de 83 %. (c) L'effet exercé par le taurocholate sur le taux maximum d'hydrolyse est moins frappant. On constate toutefois qu'il l'augmente à des concentrations relativement modestes (0.2 %), mais qu'à des concentrations supérieures (1 %), il paraît au contraire le diminuer.

TABLEAU I

INFLUENCE DU CALCIUM ET DU TAUROCHOLATE SUR LE TAUX D'HYDROLYSE

No. des essais	*Unités lipase/g de triglycérides*	*Durée (min)*	*Ca^{++}*	*Concentration en taurocholate (%)*	*Taux d'hydrolyse (%)*
1	260	15	+	0.2	58
2	400	60	+	0.2	83
3	400	180	—	0.2	42
4	4,000	180	—	0.2	83
5	400	40	+	0.0	61
6	400	40	+	0.2	80
7	200	180	+	0.0	100
8	200	180	+	0.2	100
9	200	180	+	0.6	97
10	4,000	180	—	0.0	77
11	4,000	180	—	0.2	83
12	4,000	180	—	1.0	64

Une fois que les variations du taux d'hydrolyse sont déterminées, il devient intéressant d'étudier la nature et les proportions des produits formés. La question la plus importante est la suivante: quand toutes les conditions sont réunies pour que la lipolyse soit en définitive totale, cette lipolyse engendre-t-elle transitoirement des glycérides partiels, ou, comme semble le suggérer le travail de BORGSTRÖM[9] donne-t-elle dès le début principalement naissance à du glycérol?

Les diagrammes des Figs. 3 et 4 indiquent les résultats obtenus, non pas en fonction du temps comme dans nos publications antérieures[2,3], mais en fonction du taux d'hydrolyse. Ce nouveau mode de représentation est certainement plus logique que l'ancien. Il permet aussi une comparaison immédiate entre les résultats de plusieurs expériences réalisées dans des conditions différentes. Des taux d'hydrolyse variant de 11 à 91 % ont été, par exemple, obtenus en changeant les quantités de lipase, son état de pureté, la concentration du taurocholate et la durée des essais. On voit que tous ces éléments n'affectent en rien les caractères fondamentaux du phénomène, puisque les points se placent le long de courbes régulières.

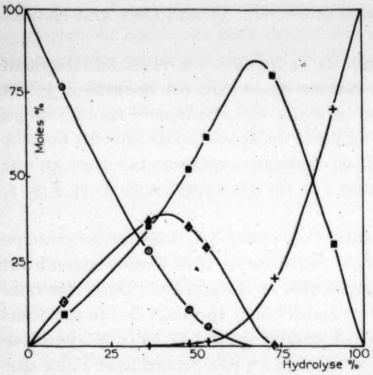


Fig. 3. Les trois étapes de la lipolyse des glycérides (essais en présence de calcium). Les proportions des divers constituants ont été calculées comme d'habitude en partant du principe que l'hydrolyse de 100 moles de triglycérides peut engendrer au maximum 100 moles de diglycérides, 100 moles de monoglycérides, 100 moles de glycérol et 300 moles d'acides. ⊙, triglycérides; ■, monoglycérides; ◇, diglycérides; ×, glycérol.

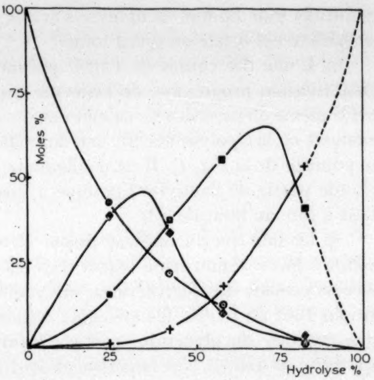


Fig. 4. Les trois étapes de la lipolyse des triglycérides (essais en l'absence de calcium). Même mode de calcul et mêmes symboles que pour la Fig. 3. Résultant d'une extrapolation peut-être inexacte, le tracé des courbes est en pointillé à partir d'un taux d'hydrolyse de 83 %.

La conclusion la plus évidente qui s'impose après examen des Figs. 3 et 4 est qu'en toutes circonstances, la lipolyse paraît s'effectuer en trois étapes successives dont les vitesses vont en décroissant. Au fur et à mesure que le taux d'hydrolyse s'élève, les triglycérides sont remplacés par des diglycérides dont les proportions sont maximum pour un taux de 40 %. Puis, viennent les monoglycérides qui, à leur tour, passent par un maximum pour un taux de 70 %. Enfin, à partir d'un taux de 70 %, les proportions du glycérol libre augmentent très vite. Les résultats sont analogues en présence et en l'absence de calcium. En l'absence de calcium toutefois, nous n'avons pas pu dépasser expérimentalement un taux d'hydrolyse de 83 %, bien que les quantités de lipase aient été portées à 4,000 unités/g. Des difficultés expérimentales empêchant d'aller au-delà, nous ne pouvons pas affirmer que les lipolyses *in vitro* en l'absence de calcium peuvent réellement être totales. Les courbes de la Fig. 4 ont donc été terminées en pointillé. Il est clair néanmoins qu'un taux d'hydrolyse de 83 % implique déjà la formation de beaucoup de glycérol libre.

DISCUSSION

Considérés dans leur ensemble, les résultats précédents suggèrent quelques réflexions:

1. Il semble tout d'abord que les divergences d'opinions concernant le caractère plus ou moins complet de la lipolyse *in vitro* aient été dues, dans une large mesure, à l'ignorance où se trouvaient les auteurs des quantités de lipase qu'ils mettaient en jeu au cours de leurs expériences. Le taux maximum d'hydrolyse susceptible d'être atteint *in vitro* dépend en premier lieu des quantités de lipase utilisées par rapport aux quantités de triglycérides à hydrolyser (Figs. 1 et 2; Tableau I). Quand ces

quantités sont faibles, la lipolyse s'arrête prématurément. Quand elles sont élevées, la lipolyse est totale ou quasi totale.

2. L'une des causes de l'arrêt prématuré de la lipolyse est vraisemblablement l'inactivation progressive de l'enzyme qui, comme on le sait, est instable à 37° et pH 8 même en présence de calcium et de son substrat. On remarquera en effet qu'au moment où la lipolyse fléchit, une nouvelle addition de lipase la fait repartir (courbe en pointillé de la Fig. 1). Il est d'ailleurs facile de constater expérimentalement qu'une grande partie de l'activité lipasique a disparu à la fin des expériences de la Fig. 1, c'est à dire au bout de 3 h.

3. Le fait que la lipolyse puisse être totale ne paraît pas affecter le principe maintes fois reconnu selon lequel cette lipolyse s'effectue en trois étapes engendrant successivement des diglycérides, des monoglycérides et du glycérol. Dans nos conditions tout au moins, les mélanges engendrés contiennent toujours des proportions très notables de glycérides partiels, sauf évidemment quand le taux d'hydrolyse approche de 100 %. Nos expériences sont d'autre part en bon accord avec l'idée que la vitesse des trois réactions successives décroît dans l'ordre indiqué ci-dessus. On voit en effet que: (a) les monoglycérides disparaissent moins vite que les diglycérides puisque leur maximum est plus élevé (Figs. 3 et 4); (b) les proportions de glycérol ne commencent à devenir notables qu'au moment où le taux d'hydrolyse dépasse 70 %; (c) la forme de la courbe de la Fig. 2 indique que l'hydrolyse est d'autant plus difficile qu'elle est plus poussée. En somme, compte tenu de son inactivation progressive, la lipase ne peut faire face à des difficultés croissantes que si elle est relativement abondante au début de l'expérience. Dans le cas contraire, les quantités restantes deviennent vite incapables de mener l'hydrolyse à son terme.

4. Le rôle des ions calcium et du taurocholate mérite également quelques commentaires. Le calcium n'est pas un véritable "activateur" de la lipolyse car il n'augmente pas sa vitesse initiale[14]. Il n'a pas non plus d'influence spécifique sur le déroulement de la lipolyse puisque les diagrammes des Figs. 3 et 4 sont pratiquement superposables. Par contre, le Tableau I nous apprend que sa présence permet à la lipolyse d'aller plus vite et plus loin avec des quantités moindres de lipase. Cet effet non spécifique nous paraît dû, en grande partie tout au moins, au fait que la formation de savons de calcium insolubles débarrasse l'interface des savons solubles qui l'encombrent. Le pouvoir inhibiteur considérable des savons d'ammonium est mis en évidence par le fait que les lipolyses sans calcium au cours desquelles le pH est maintenu à 8.0 au moyen d'ammoniaque, sont extrêmement lentes. Il est donc permis de penser que les savons de sodium sont aussi des inhibiteurs pour des raisons purement stériques et que le calcium favorise la lipolyse en les éliminant.

Par contre, une publication prochaine montrera que le taurocholate est un véritable activateur. Dans les circonstances les plus favorables, il est capable de quadrupler la vitesse initiale de la lipolyse[14]. Notons simplement pour l'instant que l'action de ce taurocholate peut être favorable ou défavorable selon la concentration (expériences 10–12 du Tableau I).

5. Enfin, l'influence décisive exercée par la valeur du rapport lipase/triglycérides sur le taux maximum de lipolyse *in vitro* suggère que la nature des produits pénétrant dans la muqueuse, et par conséquent le mécanisme de la resynthèse intervenant dans cette muqueuse, dépendent de l'abondance relative à un moment donné de la sécrétion externe du pancréas et des triglycérides en provenance de l'estomac. Si l'arrivée des

triglycérides dans le duodénum est importante et si la sécrétion pancréatique est faible ou pauvre en lipase, on peut penser que les produits absorbés sont principalement des glycérides partiels et des acides. Dans le cas contraire, ces produits doivent être principalement du glycérol et des acides. Si cette image est exacte, les théories en apparence opposées de PFLÜGER-VERZAR[15] et de HOPPE SEYLER-FRAZER[16] concernant l'absorption intestinale des glycérides se trouveraient réconciliées.

RÉSUMÉ

1. La lipolyse *in vitro* des triglycérides est contrecarrée par les difficultés croissantes de l'hydrolyse et l'inactivation progressive de la lipase. Le taux maximum d'hydrolyse susceptible d'être atteint dépend donc en premier lieu des quantités d'enzyme par rapport au substrat. En augmentant les proportions initiales de la lipase, on constate que la lipolyse tend à devenir totale.

2. Même quand elle est finalement totale, la lipolyse engendre de façon transitoire des quantités considérables de glycérides partiels.

3. Le rôle "activateur" communément attribué aux ions Ca^{++} et aux sels biliaires pendant la lipolyse est discuté.

4. Quelques incidences possibles de ces observations *in vitro* sur la nature des produits absorbés par la muqueuse intestinale et sur le mécanisme de la resynthèse des triglycérides dans cette muqueuse, sont brièvement envisagées.

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PREPARATION AND SOME PROPERTIES OF A SOLUBLE RIBONUCLEIC ACID FROM YEAST*

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SUMMARY

A method for the preparation of a soluble RNA of yeast which is capable of combining with L-leucine in the presence of ATP and enzymes has been described. The procedure involved phenol extraction of RNA from the pH 5.1 supernatant of the 78,410 × g supernatant from the cells, followed by its absorption and desorption from Ecteola-cellulose. The RNA obtained revealed considerable homogeneity in an electrophoretic field and sedimentation analyses gave a sedimentation constant $s^0_{20} = 4.0$. Comparative studies of the RNA with that prepared from ribonucleoprotein particles indicated that the soluble RNA has a distinct nucleotide composition characterized by the presence of pseudo-uridylic acid. Some other macromolecular properties of this RNA in solution were also investigated.

INTRODUCTION

In a previous paper[1], it was reported that in yeast cells the bulk of the RNA which is capable of combining with L-leucine is localized in the fraction soluble at pH 5.0 or pH 5.3 of the 105,000 × g supernatant of the homogenate. The RNA extracted from the acid-supernatant fraction was contaminated with polysaccharides which have however little effect on the [^{14}C]-leucine incorporation into RNA under the conditions previously described[1]. We have recently succeeded in purifying the RNA by means of Ecteola-cellulose, and have investigated some of its properties.

Preliminary accounts of some of the results described in this paper have been published elsewhere[2].

MATERIALS AND METHODS

Organisms: Late log-phase cells of *Saccharomyces cerevisiae*, Strain Kaneka, grown in a synthetic medium were used. For a report of the culture conditions, see the preceding paper[1].

Ecteola-cellulose: Ecteola-cellulose resin was prepared by the method of PETERSON AND SOBER[3]. The cellulose powder used was a product of "Genuine Whatman", W. and R. Balston Ltd., England.

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* Soluble RNA (S-RNA) refers to any RNA which binds amino acid in the presence of amino acid activating enzymes, amino acids and ATP.

Electrophoresis: A Tiselius electrophoresis apparatus was used unless otherwise specified. All electrophoretic analyses were performed in 0.1 *M* NaCl–0.03 *M* Tris buffer at pH 7.1 in a field of 7.3 V/cm at 3°.

Sedimentation: Sedimentation analyses were performed in a Spinco E analytical centrifuge in 0.1 *M* NaCl–0.03 *M* Tris buffer at pH 7.1.

Viscosity: An Ostwald type of viscosimenter was used. All determination were made at 20°.

U.V. absorption: Curves were determined at room temperature (*ca.* 25°) with a Beckman Model DU spectrophotometer.

Nucleotide composition: RNA was hydrolyzed in 1 *N* KOH at 37° for 24 h. After neutralization with 60 % perchloric acid, the individual mononucleotides were separated on Dowex-1-formate column as previously described[1,4].

PREPARATION OF RNA

Preparation of crude RNA from the pH 5.1 supernatant of the 78,410 × g supernatant fraction

The procedure described in a previous paper[1] was adopted with some modifications. About 100 g yeast cells (wet weight) were homogenized with quartz sand for 20 min in a hand mortar. The homogenate was suspended in 200 ml CHAO–SCHACHMAN's medium[5] and centrifuged at 6,000 × *g* for 2 min. The sediment was washed once more with 200 ml of the medium and the supernatants were combined. The solution was then centrifuged for 120 min at 78,410 × *g* in a Spinco L ultracentrifuge (Rotor No. 30). The supernatant was then converted to pH 5.1 with 1 *N* acetic acid, and immediately centrifuged at 14,000 × *g* for 10 min in a Servall SS-1 centrifuge. The pH of the clear supernatant obtained was brought back to 6.8 with 0.5 *N* KOH, and then mixed with an equal volume of 90 % commercial phenol[6] (w/v). All the operations described above were carried out at 0–2°. The emulsion was stirred for 1 h at room temperature (16–22°) with a mechanical stirrer. It was then centrifuged in the cold at 16,300 × *g* (Lourdes Centrifuge, Volume rotor) for 30 min. After the aqueous layer had been carefully pipetted out, the remainder was mixed with 300 ml cold water and centrifuged as above. The combined aqueous layers were centrifuged at 16,300 × *g* for 30 min to remove insoluble matter. Two volumes of ethanol were added to the still turbid solution in the presence of 2 % potassium acetate[6], and the crude RNA was precipitated at —5° over 18 h. The pellet formed was homogenized with about 100 ml of cold water in a glass homogenizer, and centrifuged for 20 min at 78,410 × *g* for 30 min. The clear supernatant was then dialyzed for 5 h against 3 l of cold water. The solution was centrifuged again at 14,000 × *g* for 20 min, and the clear solution was lyophilized (or was used directly for the purification of S-RNA).

The crude sample of RNA obtained was contaminated with polysaccharide to a considerable extent, although this has little effect on the ^{14}C-leucine incorporation into RNA. On electrophoretic examination, polysaccharide stayed at the starting point.

Purification of the S-RNA with Ecteola-cellulose

Crude preparations of RNA derived from 3–5 runs (total absorbancy at 260 mμ through 1 cm optical path = *ca.* 2,500) were dissolved in 100 ml of the cold 0.001 *M* potassium phosphate buffer at pH 7.1. After removal of insoluble matter by centrifugation, the clear supernatant was mixed with 7.5 g Ecteola-cellulose which had been washed well with the phosphate buffer. The mixture was shaken by hand for 5 min in an ice bath. About 90 % of the u.v. absorbing substance originally present in the crude RNA preparation was absorbed onto the Ecteola, while all the polysaccharides remained uncombined. The Ecteola–RNA complex was washed 3 times with the phosphate buffer to remove any unabsorbed material. The sediment was then mixed with 100 ml of 0.3 *M* $NaClO_4$ at pH 7.1, and centrifuged. The extraction was repeated once more. At this step, the bulk of the u.v. absorbing substances were separated from the Ecteola. The combined extract was centrifuged at 14,000 × *g* for 10 min to remove a small amount of the Ecteola particles. The RNA was precipitated from 67 % ethanol at −20° for 1 h. The precipitate was then dissolved in about 10 ml of water and dialyzed against cold water for 4 h and lyophilized. In Table I, the results of a small-scale recovery experiment are recorded.

The preparation thus obtained was a white powder, and easily soluble in water or salt solutions. No precipitation occurred in 2 *M* NaCl solution in sharp contrast to the case of the high molecular RNA derived from ribonucleoprotein particles. At this stage, electrophoretic examination indicated that contaminating polysaccharides were not detected. A small peak was however detected just ahead of the main RNA

TABLE I

RECOVERY OF U.V. ABSORBING SUBSTANCES IN ECTEOLA TREATMENT OF CRUDE S-RNA

Step	*Total absorbancy at 260 mμ*
Original crude RNA preparation	452
Not absorbed by Ecteola	63.5
Extracted with 0.3 *M* $NaClO_4$, pH 7.1	320**
Not extracted with 0.3 *M* $NaClO_4$, pH 7.1 (by difference)	68.5

1.5 g Ecteola-cellulose used.

* Measured in a cell of 1 cm optical path.
** Completely recoverable by ethanol precipitation.

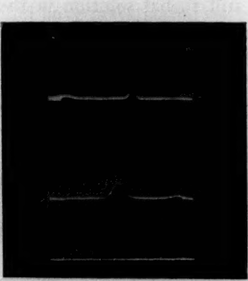

Fig. 1. Electrophoretic patterns of purified S-RNA (Prep. No. S-2): 0.6 % solution in 0.1 *M* NaCl–0.03 *M* Tris buffer, pH 7.1; photographs taken after 60 min: top, ascending pattern; bottom, descending pattern.

peak. The fast component, which presumably consisted of breakdown products of RNA, could be completely removed by a further 24 h dialysis against water or 0.1 *M* NaCl–0.03 *M* Tris buffer at pH 7.1. Thus an electrophoretically homogeneous sample of RNA was obtained (Fig. 1). The RNA prepared was usually monodispersed in the sedimentation analyses (Fig. 3).

The N/P ratio of the product was 1.63. The DNA contamination of the most pure sample was around 0.2 % relative to RNA as determined by microbiological assay of deoxyribonucleosides using *Lactobacillus acidophilus* R-26[7]*.

Comment: A rapid Ecteola treatment seems to be essential for the successful preparation of S-RNA. Extensive degradation and inactivation of RNA was observed when column chromatographic fractionation of the RNA on Ecteola was tried. RNA prepared from ribonucleoprotein particles was also absorbed onto Ecteola, but could not be removed with 0.3 *M* $NaClO_4$.

The amount of contaminating DNA in the S-RNA preparation was usually less than 1 %. It was however found that one preparation (Prep. No. S-5) contained about 4 % of DNA. Because of the serious effect of the contaminating DNA on the viscosity

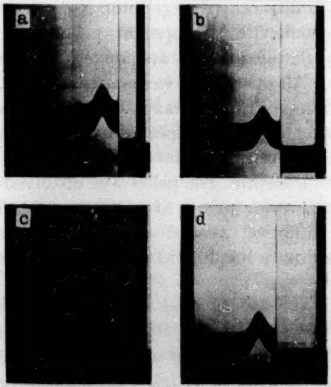

Fig. 2. Sedimentation patterns of S-RNA, H-RNA, and H-RNAhe: a, S-RNA (Prep. No. S-5) contaminated by DNA; b, same preparation as "a" after treatment with deoxyribonuclease (Prep. No. S-5b); c, H-RNA (Prep. No. H-3); d, H-RNAhe (Prep. No. H 3 he). All in 0.1 *M* NaCl–0.03 *M* Tris buffer, pH 7.1; photographs taken approximately 32 min (a), 40 min (b), 20 min (c) and 48 min (d) after reaching 56,900 rev./min; bar angle: a, 60°; b, 70°; c, 75°; d, 75°.

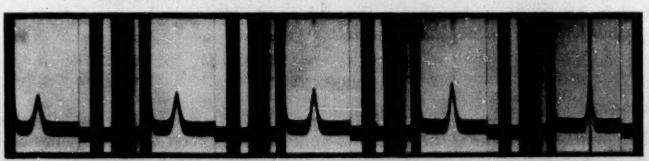

Fig. 3. Sedimentation patterns of purified S-RNA (Prep. No. S-2): 1.6 % solution in 0.1 *M* NaCl–0.03 *M* Tris buffer, pH 7.1; photographs taken at 8 min intervals at 56,900 rev./min and 24°; bar angle, 80°; synthetic boundary cell (Photograph by Dr. M. TAKANAMI).

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* Generously performed by Drs. R. and T. OKAZAKI of our institute.

behavior of the S-RNA preparation, it is desirable to remove DNA from such preparation by deoxyribonuclease. Contaminating DNA appeared as a small peak (about 8 S) in the sedimentation analyses (Fig. 2a). About 0.5 mg of pancreatic deoxyribonuclease (Worthington Biochemical Corp., U.S.A.) was added to 5 ml of 0.3 % solution of the same sample as shown in Fig. 2a in 0.001 *M* potassium phosphate buffer (pH 7.1) plus 0.001 *M* $MgCl_2$. The mixture was dialyzed against 3 l of the same solvent in the cold for 4 h. It was then treated with 3 g Ecteola as described above. The RNA released from Ecteola with 0.3 *M* $NaClO_4$ was recovered by ethanol precipitation. The sedimentation pattern shown in Fig. 2b revealed that the 8 S component had been completely removed without any effect on the main S-RNA peak.

Preparation of RNA from ribonucleoprotein particles

About 170 g yeast cells (wet weight) were ground with quartz sand for 20 min. The homogenate produced was suspended in 200 ml CHAO–SCHACHMAN's medium[5] and centrifuged at 6,000 × *g* for 20 min. The sediment was washed with 200 ml of the medium. The combined supernatants were centrifuged for 20 min at 12,000 × *g* in a Servall SS-1 centrifuge. The supernatant was then centrifuged for 120 min at 78,410 × *g* in a Spinco L ultracentrifuge (Rotor No. 30). After decanting the clear supernatant, a slightly yellowish, transparent pellet was collected. It was then homogenized with 80 ml distilled water, and mixed with an equal volume of 90 % commercial phenol (w/v). All operations were carried out at 0–2°. The mixture was stirred for 1 h at room temperature (16–22°), centrifuged in the cold at 16,300 × *g* for 1 h, and then the water layer was pipetted out. The remainder was mixed with 50 ml of cold water and centrifuged as above. The RNA was precipitated from 67 % ethanol and 2 % potassium acetate. The pellet was dissolved in 100 ml of cold water and centrifuged at 78,410 × *g* for 30 min to remove some turbid materials. The RNA was again precipitated as above, washed with 70 % ethanol 3 times, dissolved in 30 ml of cold water and finally lyophilized. This sample was designated as H-RNA (high molecular RNA).

Preparation of heat-degraded H-RNA

The method described by TAKANAMI[8] was followed for the preparation of heat-degraded H-RNA. About 100 mg of H-RNA preparation were dissolved in 20 ml 0.2 *M* NaCl, and heated at 90° for 1 h. During the heat treatment, a small amount of contaminating material (presumably proteins) was precipitated. After cooling, the solution was centrifuged at 10,000 × *g* for 10 min to obtain a clear solution. The RNA was precipitated with 2 volumes of ethanol, washed with 70 % ethanol 3 times, dissolved in 10 ml of cold water and lyophilized. The sample was called hereafter H-RNAhe.

RESULTS

Sedimentation

The conditions of the sedimentation analyses are given in the legends of Fig. 2 and Fig. 3. As may be seen in the photographs, the S-RNA preparation is usually quite homogeneous, giving a sedimentation constant of $s^0_{20} = 4.0$ S extrapolated from the values obtained at three sample concentrations (Fig. 4). This value is rather close to the mean sedimentation constant of rat liver S-RNA (3.45 S) given in a preliminary note of GOLDTHWAIT[10], but is considerably higher than that of HOAGLAND *et al.*[11] for the S-RNA of the pH 5 enzyme fraction of ascites tumor (1.85 S). The molecular weight of our RNA preparation calculated from the sedimentation constant and the intrinsic viscosity ($[\eta] = 0.05$) by the MANDELKERN–FLORY equation[9] is around 23,000, assuming a partial specific volume = 0.58, and a flexible-chain molecular model (Table II). In a preliminary note on this study[2], it was thought reasonable to assume that the molecular shape of S-RNA is a rigid rod because of the relatively small sedimentation constant and the low intrinsic viscosity value in salt solution. However, it is now found that the viscosity behavior in salt and in water (described in the next section) rather conforms to a flexible chain molecule of S-RNA.

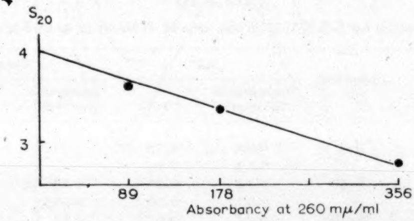

Fig. 4. Concentration dependency of sedimentation constant of S-RNA (Prep. No. S-2).

Similar experiments were also carried out with H-RNA preparations as well as with H-RNAhe for comparison. As seen in Fig. 2c, H-RNA preparations gave three boundaries, the main peak giving the sedimentation constant of 22 S and the two small peaks 19 S and 4–5 S respectively. In this case, the sedimentation constants were not extrapolated to zero concentration. It was found that the proportion of the 22 S to the 19 S component varied to some extent in different preparations. Fig. 2d shows a sedimentation pattern for H-RNAhe.

As observed by TAKANAMI[8] on rat liver ribosomal RNA, the treatment of H-RNA by heat yielded a monodisperse peak of about 4 S. The molecular weight calculated from the sedimentation constant and intrinsic viscosity was about 27,000, comparable to that of S-RNA (Table II).

TABLE II

MOLECULAR WEIGHTS OF S-RNA, H-RNA AND H-RNAhe

All measurements were carried out in 0.1 *M* NaCl–0.03 *M* Tris buffer, pH 7.1.

RNA	*Preparation No.*	S^0_{20}	*[η] (g/100 ml)*	*Molecular* weight*
S-RNA	S-2	4	0.050	23,000
H-RNA	H-3	(22**)	0.54	(1,100,000)
H-RNAhe	H-3he	4	0.057	27,000

* Calculated from the equation of MANDELKERN AND FLORY[9].
** Main component only. No correction for concentration dependency was made.

Viscosity

Samples were dialyzed overnight against a cold medium. In 0.1 *M* NaCl–0.03 *M* Tris buffer at pH 7.1, the viscosity of S-RNA was found to be much lower than that of the H-RNA. In both cases, no definite concentration-dependency was observed (Table III).

A remarkable difference between S-RNA and H-RNA was however found when the measurements were made in water as shown in Table III. In the case of H-RNA, the viscosity in water was much higher than in the salt solution and showed a considerable concentration-dependency (*cf.* ref. 12). With a concentration of 0.06 g RNA/100 ml, for example, the viscosity in water is about 22 times as much as that in salt solution. By contrast, the viscosity of S-RNA of a comparable concentration in water was only about 3.8 times as much as that in salt solution.

TABLE III

REDUCED SPECIFIC VISCOSITY OF S-RNA, H-RNA AND H-RNAhe IN SALT SOLUTION AND IN WATER

RNA	*Preparation No.*	*Salt**		*Water*	
		concentration (g/100 ml)	*η (sp./C)*	*concentration (g/100 ml)*	*η (sp./C)*
S-RNA	S-2	0.22	0.05		
		0.13	0.05		
S-RNA	S-5b**	0.14	0.068	0.13	0.21
		0.07	0.068	0.065	0.26
H-RNAhe	H-3he	0.24	0.057	0.13	0.27
		0.12	0.057	0.065	0.32
H-RNA	H-3	0.28	0.54	0.37	3.5
		0.13	0.54	0.22	4.7
		0.065	0.51	0.11	7.4
		0.033	0.59	0.055	11.3
				0.028	17.6

* 0.1 M NaCl–0.03 M Tris buffer, pH 7.1.
** Treated with deoxyribonuclease. Original S-5 preparation was contaminated with DNA. See "Comment" under "Preparation of RNA" in text.

The results obtained for the H-RNA are in harmony with those reported by other workers[12,13], suggesting that the RNA in solution exists in the form of a randomly-coiled flexible chain. Viscosity measurements on S-RNA indicated that the molecule in solution is also flexible to a considerable extent, although the difference of the viscosity of S-RNA in salt solution from that in water is much less as compared with H-RNA. The apparent less electroviscous behavior of S-RNA does not necessarily mean that the S-RNA is less flexible than H-RNA, but is rather due to the much smaller molecular size of S-RNA. In this connection, it is noteworthy that the H-RNAhe behaved in a similar way as the S-RNA.

U.V. absorption spectra

The u.v. absorption spectra of the S-RNA and that of the H-RNA were examined under three different conditions as shown in Figs. 5 and 6, and Table IV. The main differences of the spectra of S-RNA from those of H-RNA can be summarized as follows: (1) a slightly lower $\varepsilon(P)$ value around the region at 260 $m\mu$ under the three conditions studied, and (2) a considerable flattening of the top of the maximum in degraded samples especially in 0.5 N KOH. Such spectral differences should be at least in part due to the compositional differences between S-RNA and H-RNA. A higher content of cytidylic acid in S-RNA could explain the shift of spectrum

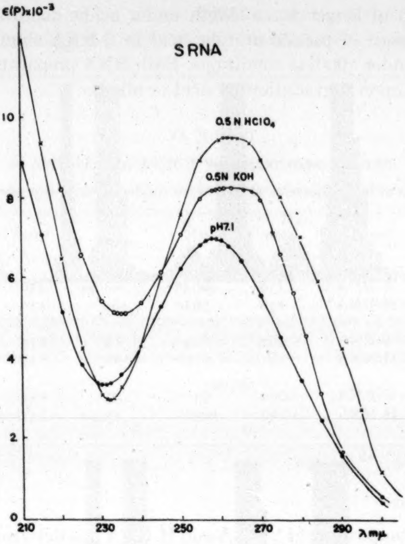

Fig. 5. U.V. absorption spectra of S-RNA (Prep. No. S-2) in 0.1 *M* NaCl–0.03 *M* Tris buffer at pH 7.1, in 0.5 *N* KOH and in 0.5 *N* $HClO_4$.

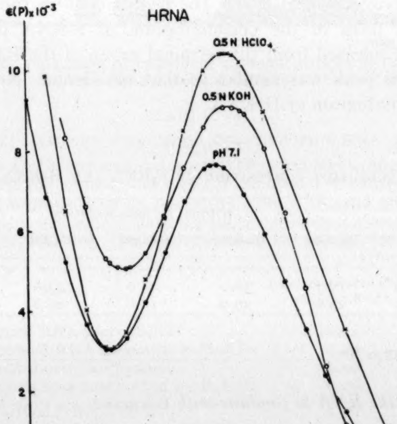

Fig. 6. U.V. absorption spectra of H-RNA (Prep. No. H-1) in 0.1 *M* NaCl–0.03 *M* Tris buffer at pH 7.1, in 0.5 *N* KOH and in 0.5 *N* $HClO_4$.

toward the direction of longer wave length under acidic conditions. Similarly, the predominant occurrence of pseudo-uridylic acid in S-RNA should cause a shift in the same direction under alkaline conditions. Both RNA preparations show a similar hyperchromic effect upon degradation by acid or alkali.

TABLE IV

OPTICAL PROPERTIES OF S-RNA AND H-RNA

Measurements unless otherwise stated were made at room temperature (25°).

Condition	*Sample*	*λmax.*	*$\varepsilon(P)$*	*% increase*	*λmin.*	*$\varepsilon(P)$*	*% increase*
0.1 M NaCl–0.03 M Tris, pH 7.1	S-RNA*	258	6948	—	230	3330	—
	H-RNA**	258	7620	—	231	3100	—
0.5 N KOH	S-RNA	262	8240	18	234	5000	51
	H-RNA	261	9120	20	235	5310	70
0.5 N HClO$_4$, 70°, 20 min	S-RNA	262	9400	35	232	2870	—13
	H-RNA	261	10300	35	232	3080	— 0.7

* Preparation No. S-2; ** No. H-1.

Nucleotide composition

The nucleotide composition of S-RNA and H-RNA was determined on a Dowex-1-formate column. As seen in Table V, Figs. 7 and 8, the composition of S-RNA is characterized by the presence of a considerable amount of pseudo-uridylic acid, and by a higher cytidylic acid and lower uridylic and adenylic acid contents when compared with the composition of H-RNA. The results confirm those of the preceding papers[1,14]. The first peak in the chromatogram of S-RNA probably represents adenosine which was released from the terminal group of the RNA by alkali[15]. The u.v. spectrum of this peak was similar to that of adenine. No extra peaks were detected in the chromatogram of H-RNA.

TABLE V

NUCLEOTIDE COMPOSITION OF S-RNA AND H-RNA

Sample	*Moles per 100 moles nucleotides*				
	Adenylic	*Guanylic*	*Cytidylic*	*Uridylic*	*Pseudo-uridylic*
S-RNA (S-4)*	19.7	30.0	23.0	22.0	5.4
H-RNA (H-1)*	25.6	29.0	18.6	26.8	trace

* Preparation No.

Test for the ability of the RNA to combine with L-leucine

In Table VI is shown the result of an experiment in which the ability of the crude and purified S-RNA preparations to combine with L-leucine was tested under the conditions described previously[1]. The purified S-RNA combined L-leucine in the proportion of 2.86 mμM per mg RNA. The value was about 40% higher than that of the crude S-RNA preparation. The fast moving component (see p. 113) was isolated

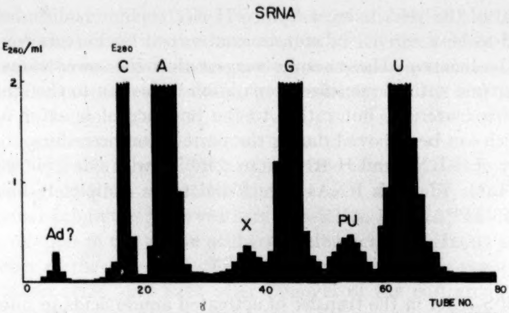

Fig. 7. Chromatographic separation of individual mononucleotides of S-RNA (Prep. No. S-4): Ad(?) = adenosine?; C = cytidylic acid; A = adenylic acid; X = unidentified; G = guanylic acid; PU = pseudo-uridylic acid; U = uridylic acid.

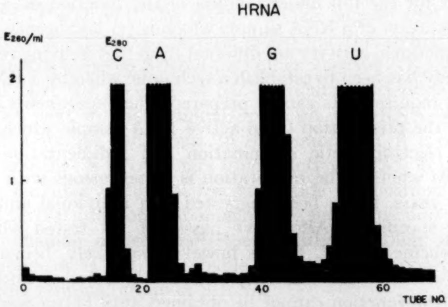

Fig. 8. Chromatographic separation of individual mononucleotides of H-RNA (Prep. No. H-1). For symbols, see Fig. 7.

TABLE VI

INCORPORATION OF [^{14}C]-L-LEUCINE INTO CRUDE AND PURIFIED S-RNA, H-RNA AND H-RNAhe

The system contained 0.2 ml of test material, 5 μM ATP, 3 μM $MgCl_2$, 50 μM Tris buffer (pH 7.6), 0.3 ml of dialyzed yeast cell extract, and 0.41 μM L-leucine (^{14}C-labeled) in a total volume of 1 ml (*cf.* ref. 1). After incubation for 15 min at 30°, the RNA (and protein) was precipitated with cold 67% ethanol–0.5 M NaCl[16], washed with this solution 4 times, with absolute ethanol twice, with ether twice, and dried. The radioactivity of the powder was measured as described previously[1].

Samples	*L-leucine linked to RNA mμM/mg RNA*
Crude RNA preparation	2.02
Purified RNA preparation (S-2)*	2.86
Fast moving component	0.00
Substances unabsorbed by Ecteola	0.00
Purified S-RNA (S-8)*	2.90
Purified S-RNA + 0.5 μM CTP	2.55
H-RNA (H-3)*	0.00
H-RNA + 0.5 μM CTP	0.00
H-RNAhe (H-3 he)*	0.00
H-RNAhe + 0.5 μM CTP	0.00

* Preparation No.

by the removal of the peak using a Spinco H electrophoresis-diffusion apparatus*, and was found to be inactive. Substances unabsorbed by Ecteola (see p. 112) were also found to be inactive. These results suggest that the lower value of the crude S-RNA as compared with the purified preparation is due not to the inhibitory action of contaminating materials, but rather to the presence of inactive u.v. absorbing substances which can be removed during the purification procedure.

The ability of H-RNA and H-RNAhe to combine with L-leucine was also tested. As shown in Table VI, both RNAs were found to be completely inactive even in the presence of CTP[15].

DISCUSSION

The function of S-RNA in the transfer of activated amino acids to microsomal ribonucleoprotein particles has been related to the presence of a specific terminal -pCpCpA group[15], and to the occurrence of pseudo-uridylic acid in the molecule[1]. One of the basic requirements for the full understanding of the function on a structural basis would be the preparation of a RNA sample which is (1) homogeneous, (2) unaltered, and (3) having a functional activity not different from that in living cells. The principal purpose of this study has been to establish a technique whereby a RNA sample which satisfies the above requirements can be prepared. The experiments described in this paper have led to the preparation of an active RNA sample which is homogeneous when judged by electrophoretic examination and sedimentation analyses. It is however not known whether the preparation is homogeneous with respect to other criteria. In recent years, it has been suggested that individual amino acids link to the corresponding specific RNA[16-18]. We have not yet tested whether the RNA obtained here is leucine-specific. This is however not likely, because the choice of L-leucine for the activity test was only incidental. Detailed information on the structural basis of the function cannot be obtained until better fractionation of the preparation can be made. The "authenticness" of our sample is also not known. However, a comparison of various molecular properties of the S-RNA preparation and of H-RNA or of H-RNAhe seems, at the present state of development in this field, to have a considerable significance for understanding the nature of the active and inactive molecules.

It has recently been suggested that the high molecular RNA prepared by phenol or detergent methods from animal microsomes or from ribonucleoprotein particles may be composed of smaller sub-units[8,12]. This suggestion stems from the preparation of a "stabilized RNA" by heating high molecular RNA[8,12,19]. We have also prepared such a RNA (H-RNAhe) by heating a solution of H-RNA obtained from yeast ribonucleoprotein particles. If the above explanation is correct, good agreement between the sedimentation constant of S-RNA and that of H-RNAhe could be taken to indicate that the basic unit of all RNA's would have an S value around 4.

The extent of the electroviscous effect in S-RNA is much less than that in H-RNA. As the molecular weight of S-RNA is estimated to be about one fiftieth of that of H-RNA, difference of viscosity behavior between S-RNA and H-RNA does not necessarily show that the H-RNA is much more flexible than S-RNA. The probable explanation is that the S-RNA molecule exists in solution mainly in the form of randomly-coiled, flexible chains* like in the case of the H-RNA molecule. In this respect, it is of considerable interest to note that H-RNAhe behaves in a relatively similar way to S-RNA in sedimentation and viscosity measurements. In other words, the macromolecular properties in solution are similar for S-RNA and the "basic unit" (H-RNAhe) of high molecular RNA (H-RNA), so far as the sedimentation and viscosity behavior is concerned.

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* Kindly performed by Dr. K. TAKATA of our institute.

As indicated in Table V, only RNA capable of combining L-leucine can be classed as S-RNA; neither H-RNA nor H-RNAhe has such an ability. The results imply that the molecular properties observed here in S-RNA, H-RNA, and H-RNAhe have no immediate relation to the amino acid combining activity. Hecht *et al.*[15] reported that the presence of the terminal -pCpCpA group of S-RNA is essential for combination with amino acids, and even after removal of the end group from S-RNA, it is easily reformed in the presence of CTP, ATP, and the pH-5 enzymes so that the S-RNA again becomes able to combine with amino acids. In the case of H-RNAhe (and also H-RNA), the ability to combine with L-leucine is completely lacking even in the presence of CTP and ATP. The results strongly suggest that RNA's other than S-RNA have neither a -pCpCpA terminal group nor the ability to form it. What causes such differences between S-RNA and other inactive RNA's we do not know. One possibility is that the presence of one particular nucleotide or a sequence of several nucleotides next to the terminal -pCpCpA of S-RNA is required for the formation of such a specific end group. In this connection, it is of considerable importance to mention that S-RNA differs from inactive RNA's in its nucleotide compositions. The remarkable feature of the nucleotide composition of S-RNA lies in the occurrence of several additional ribonucleotides such as pseudo-uridylic acid[1,14,20], and methylated guanine nucleotides[21,22] etc. Although their significance is still completely unknown, it is not improbable that these components play a role in forming the specific terminal group of S-RNA.

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* Recent optical rotation measurements generously performed for us by Dr. A. Maeda of the Chemical Institute have shown that the $[\alpha]D$ value for S-RNA was +148°, and the rotation was abolished upon alkaline hydrolysis. If the optical rotation value really can be taken as a measure of the helical content as has been discussed by Doty *et al.*[23], the S-RNA, like H-RNA[23], has a considerable amount of helical region in the molecule.

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Short Communications

Amino acid transfer from sRNA to microsome
I. Activation by sulfhydryl compounds

Incorporation of sRNA-bound amino acid to liver microsomes was described by HOAGLAND *et al*[1] as requiring a factor contained in a particle-free liver supernatant. In attempts to study the function of this fraction, difficulties in obtaining such an effect were encountered. Although a fair amount of protein was removed by washing with aq. sucrose, the washed preparation showed no consistent effect of supernatant (Table I), and sometimes even slightly better incorporation. Ageing the microsomes at between 5–10° for about 2 h, however, caused loss of activity, most of which was recovered on addition of supernatant. Such an effect using the pH-5 precipitate of liver supernatant is illustrated in Table II which shows that nearly full activity may be regained by adding GSH. On the other hand, GSSG was quite inhibitory as can be seen in Table III using freshly prepared microsomes; this inhibition could be partly reversed by pH-5 precipitate. GSH increased transfer slightly, and pH-5 precipitate caused still further stimulation.

In a more systematic study of the SH effect, a microsome preparation was used that had been moderately aged by incubation at pH 7.4 for 30 min at 30° (Table IV).

TABLE I

EFFECT OF WASHING ON THE TRANSFER OF [^{14}C]LEUCINE FROM sRNA TO MICROSOMES

The incubation was for 3.5 min at 25°, and the reaction mixture contained about 7 mg dry wt. of microsomes. Standard conditions used in this and the following tables were as follows: the rat-liver microsomes were isolated according to LITTLEFIELD AND KELLER[2] using the 0.25 M sucrose medium, and were washed by resuspending in 0.01 M Tris–HCl, pH 7.2, 0.002 M $MgCl_2$, and 0.23 M sucrose, followed by recentrifugation at 105,000 × g for 1 h. The medium contained in a total volume of 1 ml: 0.2–0.5 mg [^{14}C]leucine-RNA, 1000–3000 counts/min; 0.003 M ATP; 0.010 M CAP; 30 μg of CAP-kinase; 0.0012 M GTP; 0.100 M Tris–HCl, pH 7.4–7.6; 0.05 M KCl; and 0.006 M $MgCl_2$. The reaction is stopped by the addition of 5 ml cold 5 % trichloroacetic acid, and the precipitate is washed twice in the centrifuge with 5 ml cold 5 % trichloroacetic acid and once with 5 ml alcohol–ether (3:1). The washed precipitate is now resuspended in 5 ml 5 % trichloroacetic acid, heated for 15 min to 90°, cooled, centrifuged, and the supernatant discarded. The precipitate is washed again with alcohol–ether (3:1), plated, and counted in a Nuclear Chicago windowless gas-flow counter.

Microsomes	*Microsomal supernatant*	*counts/min in protein*
Unwashed	—	285
Washed	—	342
Washed	+	342

Abbreviations: GSH, GSSG, glutathione and its oxidized form; SH, sulfhydryl; TPNH, reduced triphosphopyridine nucleotide; ATP, adenosine triphosphate; GTP, guanosine triphosphate; CAP, carbamyl phosphate; sRNA, soluble ribonucleic acid; Tris, tris(hydroxymethyl)-aminomethane.

TABLE II

THE INFLUENCE OF VARIOUS ADDITIONS ON THE TRANSFER OF AMINO ACIDS FROM sRNA TO AGED MICROSOMES

Incubation was for 3 min at 35°. Every tube contained: 2 μmoles GTP, 100 μmoles Tris–HCl pH 7.6, 5 μmoles $MgCl_2$, 50 μmoles KCl, 6.8 mg of microsomes protein (biuret), and 1900 counts/min of [^{14}C]leucine-RNA and 0.5 mg of sRNA. For other conditions, *cf.* Table I.

Additions	*Counts/min*
None	39
pH-5 ppt., 1 mg protein (biuret)	333
pH-5 supernatant, 4 mg protein	621
20 μmoles GSH	460

TABLE III

THE EFFECT OF GSH AND GSSG ON THE TRANSFER OF [^{14}C]LEUCINE FROM sRNA TO LIVER MICROSOMES

Incubation was for 10 min at 35°. Other conditions were the same as those listed in Table I.

Additions	*Counts/min*
None	968
GSSG, 2 μmoles	58
GSSG, 2 μmoles + pH-5 enzymes	678
GSH, 1 μmole	1210
pH-5 enzymes, 5 mg	1550

TABLE IV

THE REACTIVATION OF AGED MICROSOMES

The microsomes were aged for 30 min at 30° at pH 7.4 in air. With fresh microsomes, 968 counts/min were transferred without additions. The incubation medium is the same as that in Table I.

No.	*Additions*	*Counts/min*
1.	None	191
	pH-5 fraction, 5 mg	1002
	Cysteine, 10 μmoles	910
2.	None	111
	GSH, 1 μmole	259
	GSH, 1 μmole + GSSG reductase + 0.5 μmole TPNH + 5 μmoles glucose 6-phosphate + 5 μg Zwischenferment	798
	pH-5 enzymes, 5 mg	933

Such ageing reduced the transfer to about one-fifth, and the addition of the pH-5 fraction brought the value up to a little above the non-aged preparation; addition of an SH carrier, in this case cysteine, stimulated incorporation almost equally well. In Expt. 2 in Table IV, a similarly aged preparation with a much lower concentration of GSH was only slightly stimulated. If, however, GSSG reductase and a TPNH-generating system were added to regenerate GSH, activity was brought to nearly as high a level as with addition of pH-5 enzyme.

Further confirmation of the oxidative nature of the inactivation caused by ageing was obtained by showing that it could be considerably reduced by keeping the incubation mixture in nitrogen (Table V). The degree of inactivation by ageing was partly dependent on pH, and was greater at pH 8.5 but decreased at pH 6 as might be expected from easier oxidation of GSH at alkaline pH. It was also found that incorporation of amino acid into protein in this system was sensitive to iodoacetate, 60 % inhibition being caused by 0.001 *M* inhibitor.

From these experiments it is concluded that an SH function may be involved in the transfer reactions. It seems to be significant that the inactivation caused by relatively mild manipulation was reversed by the supernatant fraction, and that the same effect could be obtained by small amounts of GSH together with a GSH-regenerating system. Since the supernatant contains both GSH and GSSG reductase, the supernatant effect may, in part, be ascribed to the maintenance of a favorable SH level in the preparation. In view of the difficulties in obtaining a preparation that clearly showed a supernatant effect, for a while we were under the impression that the supernatant effect observed by others[1,3] might be explained by the oxidizability of the system as reversed by GSH. As will appear in the subsequent communication, however, a more specific effect has now been obtained in addition to the SH function.

TABLE V

PRESERVATION OF ACTIVITY ON AGEING IN NITROGEN

The incubation medium was the same as in Table I, to which 0.2 ml microsomes (= 0.7 g liver) and 0.1 ml 0.02 *M* Tris–maleate buffer, pH 7.5, were added. After ageing for 30 min at 30°, 0.2 ml Tris–HCl, pH 7.4, and other components were added to a final volume of 1 ml. The further procedure was that described in Table I.

Conditions	*[^{14}C]leucine transfer (counts/min)*
Air, not aged	649
Air, aged	110
N_2, aged	533

This work was carried out during the tenure of a fellowship to one of us (W. C. H.) from the Netherlands Organization for Pure Research (Z.W.O.)

The Rockefeller Institute, New York 21, N.Y. (U.S.A.)

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Amino acid transfer from sRNA to microsome
II. Isolation of a heat-labile factor from liver supernatant

Although the experiments of the previous paper[1] indicate that the supernatant effect is partly due to an SH requirement, in many experiments the liver supernatant caused an additional stimulation that could not be fully substituted by SH compounds or a GSH-generating system. A requirement for a further supernatant fraction, probably protein, will be more clearly brought out in the present paper. In order to demonstrate this requirement, a means was needed of further depleting the microsomes of stubbornly adhering material; DOC in moderate concentration had been used to purify microsomes without destroying activity[2,3]. It will be shown that washing with 0.5 % DOC yielded a preparation that needed supplementation by a heat-labile fraction of liver supernatant.

Rat-liver microsomes were prepared in LITTLEFIELD AND KELLER's medium A (see ref. 4), using 24-h fasted animals in order to deplete liver glycogen, which appears to be important. The rinsed microsomes were homogenized in 0.5 % DOC-medium A (10 ml per liver) and recentrifuged at 105,000 × *g* for 2 h. The pellets were rinsed several times with medium A, and finally homogenized in the same medium without DOC. The homogenate of such particles withstood lyophilization and storage at —20° for as long as 3 weeks with little or no loss of activity. Soluble RNA was isolated by the phenol method from briefly heated *Escherichia coli* paste. Since *E. coli* sRNA had been found to be as active as rat liver sRNA in the transfer of leucine to liver microsomal particles, the more easily available bacterial sRNA was used exclusively in these experiments. Amino acid loading was carried out with an alumina-ground *E. coli* extract as the enzyme source.

TABLE I

EFFECT OF SUPERNATANT ON DOC-EXTRACTED PARTICLES

The medium contained in a volume of 1 ml: DOC-extracted particles (2.0 mg protein); 0.24 mg sRNA loaded with amino acids, including 1500 counts/min [^{14}C]leucine (10 μC/μmole); 0.003 *M* ATP; 0.01 *M* PEP; 30 μg PEP-kinase; 0.0003 *M* GTP; 0.100 *M* Tris–HCl, pH 7.0; 0.05 *M* KCl; 0.006 *M* $MgCl_2$. After a 5-min incubation at 35°, 5 % trichloroacetic acid was added and the precipitate was extracted with 5 % trichloroacetic acid at 90° for 15 min. The precipitated protein was washed with 5 % trichloroacetic acid and with ethanol–ether (3:1), plated, and counted in a Nuclear Chicago windowless gas-flow counter.

Additions	*[^{14}C]leucine transferred counts/min*
None	5
GSH, 0.010 *M*	19
105,000 × *g* supernatant	277
GSH, 0.010 *M* + 105,000 × *g* supernatant	249

Table I presents experiments with DOC-washed particles where even in the presence of an SH compound practically no activity developed except on addition of 105,000 × *g* supernatant. The DOC wash after dialysis also stimulated, indicating that the active factor had, indeed, been eluted by this procedure. Similar supernatant

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Abbreviations: SH, sulfhydryl; DOC, desoxycholate; sRNA, soluble ribonucleic acid; GSH, glutathione; ATP, adenosine triphosphate, GTP, guanosine triphosphate; PEP, phosphoenolpyruvate; Tris, tris(hydroxymethyl)aminomethane.

stimulation could be obtained with microsomes from a mouse plasma cell tumor[5], but here washing with the usual salt medium was sufficient.

Fig. 1 shows the effect of increasing amounts of supernatant on leucine transfer. An almost complete dependence on supplement made this preparation a convenient assay system. The active component was found to be completely destroyed by heating at 60° for 5 min, and was non-dialyzable. Ammonium sulfate and acetone fractiona-

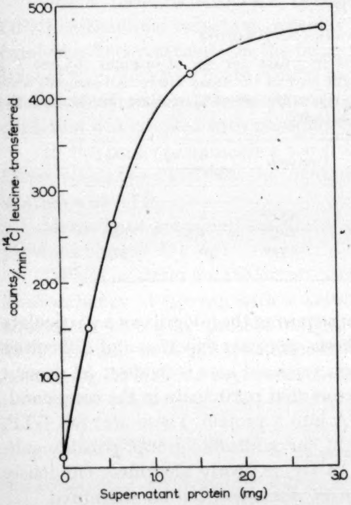

Fig. 1

Fig. 1. Dependence of leucine transfer on added 105,000 × *g* supernatant. Incubation mixture and conditions were the same as in Table I except as follows: DOC particles, 2.5 mg protein; 0.29 mg sRNA; 0.010 *M* GSH.

Fig. 2. Dependence of leucine transfer on a purified supernatant factor. Incubation mixture and conditions were the same as in Table I except as follows: DOC particles, 2.5 mg protein; 0.10 mg sRNA; 0.010 *M* GSH.

Fig. 3. Time curves of leucine transfer with different amounts of a purified supernatant factor (lower curve, 15 μg protein; upper curve, 75 μg). Incubation mixture and conditions were the same as in Table I except as follows: DOC particles, 2.5 mg protein; 0.14 mg sRNA; 0.010 *M* GSH. The solid square indicates transfer in the absence of GSH, and is paired with the upper 5-min value.

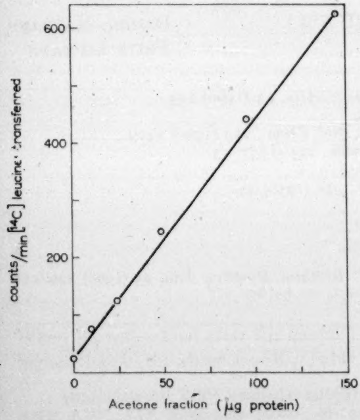

Fig. 2

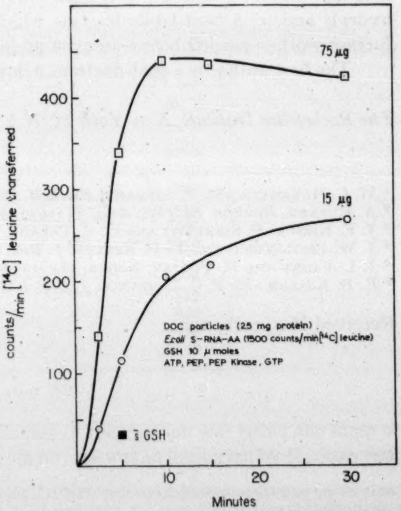

Fig. 3

tion yielded a 30-fold purification with 30% over-all recovery (Table II). Unfortunately, the purified preparation was rather labile.

Fig. 2 shows an assay of purified factor, and Fig. 3 time curves for leucine transfer at two different concentrations. In each case, 0.010 *M* GSH was present. It may be noted in Fig. 3 that the SH effect, not apparent with unfractionated supernatant (Table I), was brought out clearly with the purified fraction. Cysteine and GSH were equally effective.

TABLE II

PURIFICATION OF THE SUPERNATANT FACTOR

One unit equals counts/min [^{14}C]leucine transferred in 5 min per mg of original 105,000 × *g* supernatant protein, adjusting to values in the straight part of the assay curve. All samples were tested with the same microsome preparation. In other runs the 26–32% acetone fraction had the highest specific activity.

Fraction	*Total units*	*Specific activity units/mg*	*Recovery %*
105,000 × *g* supernatant (10 rat livers)	4370	1	
39–50% (w/v) ammonium sulfate (pH 7.0)	2650	3.3	60
20–26% acetone (pH 6.4, –6°)	1340	30	30

We conclude that the analysis of a reaction requiring the integrity of a particulate component, such as microsomal protein synthesis, presents experimental difficulties that remain to be further resolved. The progress reported here is modest. At present we can distinguish reasonably clearly three factors that participate in the compounding of the active amino acids carried by sRNA into a protein. These are: (a), GTP, long ago found by KELLER AND ZAMECNIK[6]; (b), an oxidizable group, possibly sulfhydryl; and (c) a heat-labile fraction which, so far, is poorly identified. Obviously further work is needed before an even preliminary discussion can be attempted.

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Studies on menadione reduction and reoxidation with *Azotobacter vinelandii*

The respiratory-chain preparations of *Azotobacter vinelandii*, called washed small particles (WSP) by TISSIÈRES[1–3], differ in many respects[4] from the respiratory particles obtained by disruption of mammalian mitochondria (KEILIN AND HARTREE heart-muscle preparation[5]).

One of these differences is that the *Azotobacter* particles contain a very active DPNH-menadione reductase, whereas the heart-muscle preparation is very poor in catalysing this reaction[6]. On the other hand, COLPA-BOONSTRA AND SLATER[6] demonstrated that the same heart-muscle particles were able to catalyse very actively the oxidation of reduced menadione (K_3H_2) and that in rat-heart sarcosomes this oxidation was coupled with phosphorylation.

In the present communication it is shown that also the WSP fraction of *Azotobacter vinelandii* catalyses the oxidation of K_3H_2 in a reaction coupled with the synthesis of ATP.

Methods of preparing substrates and enzyme fractions have been described in previous papers[1–7].

DPNH oxidation in the presence of WSP was measured by following the decrease in absorbancy at 340 mμ with a Zeiss spectrophotometer and expressed in terms of equivalent μl O_2/mg protein/h (Q_{O_2}). The reactions were started by adding the WSP fraction in a suitably diluted suspension to the spectrophotometer cuvette containing DPNH and the other components of the reaction mixture listed in Table I.

TABLE I

INFLUENCE OF MENADIONE ON DPNH OXIDASE OF WSP IN THE PRESENCE AND ABSENCE OF KCN

Final concentrations of reagents: DPNH, 10^{-4} *M* (only in test cuvette); phosphate buffer (pH 7.0), 0.04 *M*; ethanol, 1 % (v/v); WSP, diluted in distilled water, 3 mg protein/ml; K_3 and KCN as shown. Temperature of Expt. 1, 28.9°; of Expt. 2, 22.1°.

Expt.	K_3 (10^{-4} *M*)	*KCN* (10^{-2} *M*)	Q_{O_2}
1	—	—	3000
	—	+	0
	+	—	700
	+	+	745
	+*	—	3290
	+*	+	3010
2	—	—	1400
	+	—	1355
	+	+	835
	+*	—	2440
	+*	+	1140

* K_3 added 10–50 sec after WSP.

When K_3 was added after the enzyme the rate of the reaction was either the same or higher than in the absence of K_3 (Table I); in no case was an inhibition by K_3 observed.

Abbreviations: WSP, washed small particles; DPNH, reduced diphosphopyridine nucleotide; AMP, ADP, ATP, adenosine mono-, di- and triphosphate; K_3, 2-methyl-1,4-naphthoquinone; K_3H_2, 2-methyl-1,4-naphthohydroquinone.

When, however, K_3 was present before the enzyme was added the DPNH oxidase was sometimes inhibited considerably. This probably means that K_3 is inhibitory if the enzyme (presumably the flavoprotein) is oxidized, but not when it is reduced as is the case when K_3 is added after the enzyme. The oxidation of DPNH in the presence of K_3 at pH 7.0 is largely cyanide-insensitive (Table I).

Manometric experiments with the same type of enzyme preparation and succinate as the substrate revealed that the oxidation of succinate by K_3 was only a few percent of the succinic oxidase activity. This result is similar to that obtained with heart-muscle preparation, and shows that the K_3 reductase, which is present in the bacterial preparation but not in the mammalian, does not work with succinate as the substrate. Other examples of a DPNH–K_3 reductase have been reported by WOSILAIT AND NASON[8] in *Escherichia coli*, by WEBER *et al.*[9] in *Mycobacterium phlei*, and by ERNSTER *et al.*[10] in liver.

K_3H_2 is rapidly oxidized by the WSP fraction of *Azotobacter vinelandii* at pH 6.24 where the autooxidation of K_3H_2 is negligible[6]. The reaction was completely inhibited by 10^{-2} *M* KCN. The course of the reaction follows first-order kinetics and the K_3H_2 oxidase activity, expressed in terms of the first-order velocity constant *k* ($\text{min}^{-1} \times [\text{mg protein/ml}]^{-1}$), was 20–25 at 28°, not very different from the value of 10–15 at 22° obtained with heart-muscle preparation[6]. This is in contrast to the difference in DPNH oxidase activities of both types of preparations, the bacterial preparation being 10–15 times more active than the mammalian. Antimycin A, in concentrations much higher than necessary completely to inhibit the oxidation of DPNH, K_3H_2 or succinate by heart-muscle preparation[6], had no effect on the oxidation of these substrates by the *Azotobacter* particles. The DPNH oxidase activity of these particles was also insensitive to Amytal (1.8 m*M*). The insensitivity of *Azotobacter* preparations to antimycin is in agreement with the work of REPASKE AND JOSTEN[11] and BRUEMMER *et al.*[12].

The results presented in Table II show that oxidation of K_3H_2 by WSP is accompanied by phosphorylation, the P:O ratios varying between the narrow limits, 0.19 to 0.22. The ratio obtained with DPNH as substrate in the same experiment was sometimes the same, sometimes much higher. Thus it seems that the phosphorylative activity accompanying the oxidation of DPNH is more labile than that accompanying K_3H_2 oxidation. Oxidative phosphorylation with DPNH as substrate was uncoupled by K_3 (*cf.* PINCHOT[13], who found a similar uncoupling with *Alcaligenes faecalis*). The degree of uncoupling was not affected by pre-incubation of the WSP with K_3 for 45 min. It has been suggested[9] for *Mycobacterium phlei* that there are two different pathways for the oxidation of DPNH in the presence of K_3. Though this possibility is not excluded for the *Azotobacter* particle, it is clear from the results presented in Table II, Expts. 2 and 3, that this cannot be an explanation for the uncoupling of phosphorylation by K_3, since such a sidepath would not cause P:O ratios lower than those obtained with K_3H_2 as substrate. In this respect it must be emphasized that these experiments were carried out at pH 6.24, where the autooxidation of K_3H_2 formed from DPNH and K_3 is very low, so that any reoxidation of K_3H_2 would need an enzymic pathway. A more likely explanation is that menadione reacts with an enzyme necessary for phosphorylation, thus acting as a real uncoupling substance. This appears to be in accordance with the finding of BRODIE AND BALLANTINE[14] that vitamin K_1 can restore phosphorylation in light-inactivated preparations of

TABLE II

OXIDATIVE PHOSPHORYLATION BY THE WSP FRACTION OF *Azotobacter vinelandii* WITH K_3H_2 OR DPNH AS SUBSTRATE

The procedure has been described in ref. 3, 6. Reaction mixture: phosphate, 0.03 *M*; glucose, 0.007 *M*; ethylenediaminetetraacetate, 0.001 *M*; $MgCl_2$, 0.005 *M*; AMP, 0.0002 *M*; ADP, 0.0002 *M*; hexokinase; DPNH, K_3 and K_3H_2 as indicated. The pH was 6.24, the reaction volume 2.6 ml. Trichloroacetic acid was added after the substrate was completely oxidized and Δ (esterified phosphate) measured as described in ref. 7.

Expt.	*Substrate*		*Addition K_3 (mM)*	*P:O*
	DPNH (mM)	*K_3H_2 (mM)*		
1	0.45	—	—	0.43
	—	0.42	—	0.19
2	0.76	—	—	0.22
	0.76	—	0.1	0.08
	—	0.65	—	0.22
3	0.74	—	—	0.24
	0.74	—	0.1	0.08
	—	0.65	—	0.21
	—	0.65	0.1	0.18
4	0.68	—	—	0.49
	0.68	—	0.1	0.33
	—	0.77	—	0.22
	—	0.77	0.1	0.19

Mycobacterium phlei, and that this phosphorylation is competitively inhibited by lapachol or dicoumarol. It seems possible that the phosphorylative step(s) of the respiratory chain measured with DPNH as substrate is not the same as that measured with K_3H_2 and that the former is more susceptible to uncoupling by K_3.

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Sedimentation of myosin in urea solutions

Studies of myosin in urea solutions were first reported by SNELLMAN[1], and later in some detail by TSAO[2]. More recently KOMINZ *et al.*[3] reported the isolation of a small fragment from urea-treated myosin. The consensus of these studies is that myosin breaks up in urea to yield fragments smaller than the original molecule, presumably with concomitant loss of three-dimensional structure.

In the experiments here reported, three distinct peaks were observed in solutions of rabbit skeletal myosin in 8 *M* urea, studied in the velocity ultracentrifuge (Fig. 1). We designate them in order of increasing sedimentation rate as the α, β and γ peaks.

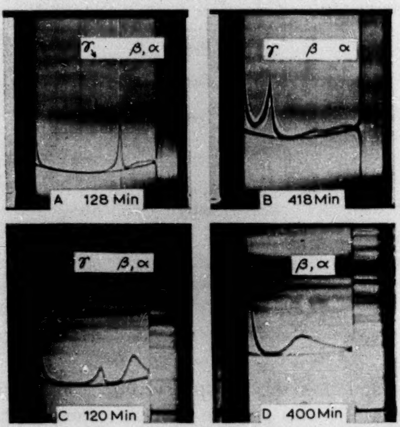

Fig. 1. A and B: Sedimentation of freshly-prepared myosin (*c*, 0.85 %) at 50,740 rev./min in solvent: 8.0 *M* urea, 0.37 *M* KCl. Double-sector cell shows solvent baseline. C and D: Sedimentation of iodoacetamide-treated myosin after one month in 8 *M* urea. Speed, 47,660 rev./min; solvent and protein concentration as in A and B. Sedimentation proceeds from right to left. Diaphragm angles: A, 70°; B, 40°; C, 65°; D, 52.5°.

Use of a double-sector cell proved indispensable in resolving the minor peaks. The γ peak comprises about three quarters of the total area under the peaks, the α and β peaks comprising about one eighth each. The relative areas do not appear to change with total protein concentration. To test whether any of the peaks could be artefacts produced, for example, by a redistribution of urea in the solution, we carried out a control sedimentation on bovine serum mercaptalbumin in the same solvent. At every stage of the sedimentation of the albumin (duration 10 h at 59,780 rev./min) only a single peak was observable, and no anomaly appeared (see also ref. 4). Plots of log peak position *vs.* time were linear both in the albumin and the myosin solutions. This relationship seems surprising in view of the obvious formation of a urea gradient during the run, but it does provide a convenient empirical description of the system.

Reporting the sedimentation coefficients poses a problem, since the usual assumptions about the density and viscosity relationships may lose significance in such a mixed solvent. For this reason, we report in Table I the actual rate of sedimentation, $S'' \equiv \frac{\mathrm{d}r/\mathrm{d}t}{\omega^2 r}$, as well as the apparent sedimentation coefficient $S'_{20,\mathrm{w}}$ obtained by making the conventional density and viscosity corrections. For the solvent used in these experiments $\varrho_{20} = 1.133$ and $\left(\frac{\eta_{20}}{\eta_{20,w}}\right) = 1.69$. Note that whereas the α and β peaks show little or no concentration dependence in their sedimentation coefficients, a large effect is seen for the γ peak. Such an extreme concentration dependence probably reflects a marked molecular asymmetry; it does not seem to be consistent with a random-coil model. It appears that the component corresponding to the γ peak may be roughly the same molecular weight as the parent myosin molecule.

TABLE I

SEDIMENTATION COEFFICIENTS OF MYOSIN COMPONENTS IN UREA

Component	*Total protein* concentration (%)*	S''	$S'_{20,w}$
α	0.85	0.4	1.2 ± 0.3
	0.43	0.6	1.6 ± 0.3
β	0.85	1.2	3.0 ± 0.3
	0.43	1.2	3.3 ± 0.3
γ	1.29	0.9	2.3
	1.29	0.8	2.1
	0.62	1.7	4.7
	0.39	2.3	6.1
	0.22	3.4	9.2
	0.12	4.3	11.4
	0.06	4.3	11.6

* Corrected, in the case of the γ-component, by a mean radial-dilution factor, $\frac{r^2\,\text{men.}}{r^2}$.

The sedimentation patterns of Fig. 1 (A and B) are qualitatively unchanged over a pH range from 3.3 to 10.5, and are also unaffected by short periods of heating (1 h at 50°) at both these extremes of pH. The pattern is further indifferent to the addition of an excess (over the $^-$SH content) of iodoacetamide or *p*-chloromercuribenzoate. Preliminary observations of the time stability of myosin in 8 *M* urea at 25° indicated no change over a period of several days. The sedimentation pattern of such a myosin solution after two weeks at room temperature is qualitatively unchanged, but the sedimentation rate of the γ peak increases, in the presence or absence of thiol reagents. After one month in 8 *M* urea at room temperature, myosin shows a markedly altered sedimentation pattern (Fig. 1 C and D). The β peak grows at the expense of the γ peak, suggesting a simple transformation of the latter to the former. Such a process also offers an explanation for the increase of the sedimentation rate of the γ peak on shorter periods of ageing, in view of its demonstrated concentration dependence. The slowness of this change is consistent with the observations of TSAO[2]. A single experiment suggests that the transformation is more rapid at 4° than at room temperature.

Following an interchange of correspondence with W. F. HARRINGTON, two double-sector sedimentations with myosin in 5 *M* guanidine hydrochloride were run. These show no γ peak, a large β peak, and a typical small α peak. Although the relative areas under the two peaks were difficult to measure, we estimate that the α peak contains about 10% of the total area. The following values for the β peak were determined: at 0.96% myosin, $S'' = 0.7_4$ and $S'_{20,w} = 1.7$; at 0.48% myosin, $S'' = 1.0_0$ and $S'_{20,w} = 2.3$. This present finding of the heterogeneity in 5 M guanidine hydrochloride is in contrast with the conclusions of KIELLEY AND HARRINGTON[5], who deduce that myosin in this solvent dissociates into three identical subunits. Trial calculations show, however, that the heterogeneity will probably not greatly alter the weight-average molecular weight found by these authors.

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Manganese content and changes in light absorption during photosynthesis in green algae

Studies on the effect of manganese deficiency on photoreduction and photosynthesis in green algae have led to the conclusion that manganese is specifically involved in photosynthetic O_2 evolution[1–4]. This assumption received further support from measurements of delayed light emission and fluorescence in manganese-deficient algae[5] and from work on the dependence of the HILL reaction on the manganese content of algae and higher plants[6]. In order to obtain further information concerning the role of manganese in photosynthesis, we have studied the effect of manganese deficiency on the changes in light absorption during illumination of green cells.

Four types of changes in light absorption have been observed during photosynthesis[7–10]. Those of Type 2 are obviously connected with the splitting of water. Their amplitude is independent of temperature, and they are not influenced by inhibition of the other basic processes of photosynthesis (*i.e.*, O_2 evolution, photosynthetic phosphorylation, and CO_2 reduction)[7,11].

Ankistrodesmus braunii (strain Marburg) was grown in culture media with and without addition of manganese[5]. After about 3 weeks, the photosynthetic activity of the manganese-deficient cells had dropped to one-fourth of that observed with normal algae. Fig. 1 shows that the changes in light absorption of Type 2, induced by short (10^{-4} sec) flashes of light[11], are not influenced at all by manganese deficiency.

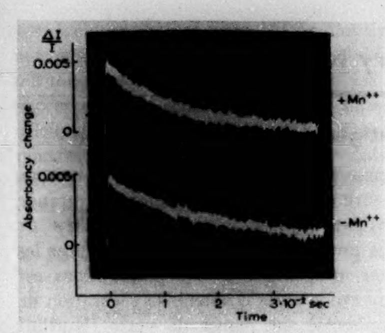

Fig. 1. Changes in light absorption of Type 2 at 515 mμ after illumination by short flashes of light (10^{-4} sec) in normal and in manganese-deficient cells. *Ankistrodesmus* suspended in phosphate buffer, pH 6.5; temp., 20°; light saturation.

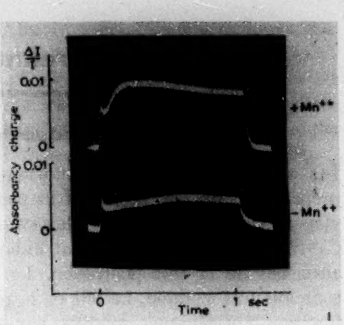

Fig. 2. Changes in light absorption of Type 2 at 515 mμ during 1 sec of illumination in normal and in manganese-deficient cells. *Ankistrodesmus* suspended in phosphate buffer, pH 6.5; temp., 20°; light saturation.

This again demonstrates that manganese does not serve as a catalyst in the water-splitting reaction of photosynthesis.

A considerable difference, however, can be observed in the second, slow phase of the changes in light absorption of Type 2, which occurs during longer periods of illumination ($>$ 0.1 sec). This second phase disappears in manganese-deficient cells (Fig. 2), just as it does in normal cells at about 0° (see ref. 8, 11). After addition of manganese, both the second phase and the normal photosynthetic oxygen evolution are again observed. It should be stressed that the loss of the second phase is brought about directly by the manganese deficiency and not indirectly by the inhibition of photosynthesis resulting from it. This follows from the observation that the second phase remains unchanged when photosynthesis has been inhibited by lack of CO_2 or by poisoning with cyanide.

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Preliminary Notes

Etude èlectrophorétique des antigénes de la transplantation

Les antigènes de la transplantation se présentent habituellement sous forme de préparations opalescentes dont ils peuvent être séparés par centrifugation à grande vitesse[1,2]. Cependant, BILLINGHAM, BRENT ET MEDAWAR d'une part[1], OTH ET CASTERMANS, d'autre part[3], ont indiqué des procédés permettant de solubiliser les antigènes de la transplantation. Le pouvoir antigénique de ces préparations est toutefois très instable. Le but de la présente étude a été d'obtenir la séparation de fractions antigéniques solubles par électrophorèse sur papier de verre (Hurlbut no. 934 – AH).

Trois types de préparations antigéniques ont été soumis à l'électrophorèse: (a) l'extrait en NaCl 0.15 M, citrate sodique 0.01 M, pH 7[2,3]; (b) la préparation obtenue par salting out au sulfate ammonique à 50 % de saturation, de la préparation A[2,3]; (c) l'extrait obtenu par gélification des cellules en eau distillée suivant BILLINGHAM, BRENT ET MEDAWAR[1]. Dans tous les cas le matériel de départ consistait en cellules spléniques et thymiques de souris de souches Albino Swiss et C57 BL. Le test d'antigénicité a consisté en l'élimination accélérée des greffes cutanées transplantées sur des animaux sensibilisés par l'antigène présumé[3]. Les électrophorèses ont été réalisées au moyen d'une cellule SPINCO C.P., permettant de recueillir 16 fractions (tampon Na_2HPO_4 0.01 M, pH 7.5; réfrigération à 0°; opération 8 à 10 h; voltage constant 500 V et intensité oscillant aux environs de 50 mA). L'amidoschwartz a été utilisé pour la coloration sur papier des protéines, et le Schiff pour la coloration des glycoprotéines. Les acides nucléiques ont été décelés au moyen de la lampe Mineralight, S.L. 2537. Des dosages colorimétriques ont été réalisés par les méthodes suivantes: (1) FOLIN-CIOCALTEU[4] pour les protéines; (2) BIAL[5] pour l'ARN; (3) H_2SO_4-orcinol[6] pour les hexoses.

L'électrophorèse des trois préparations A, B, et C a donné des résultats fort comparables. Au cours de l'expérience, apparaît une traînée verticale de coloration beige. Son intensité, maximum au point d'alimentation, se réduit progressivement vers le bas, mais atteint néanmoins le bord inférieur du papier dans la plupart des cas. Elle est constituée de matière insoluble qui est recueillie dans le tube immédiatement sous-jacent. Dans un cas, toute trace de matière insoluble a disparu avant le bord inférieur du papier, ce qui suggère que la totalité de la matière mise en expérience a été dissociée et solubilisée par le champ électrique. L'activité antigénique a été retrouvée: 1° – dans la fraction insoluble, recueillie à la verticale du point d'alimentation, dans les cas de solubilisation partielle; 2° – dans les fractions recueillies entre 15 et 23 cm de la perpendiculaire du point d'alimentation, du côté de la cathode, dans tous les cas. L'analyse élémentaire de toutes les fractions solubles actives a donné des résultats identiques; protéines, 85 %; ARN, 12 %; galactose–manose 2–3 %. Les doses injectées, donnant une réponse positive, ont été de l'ordre de 60 à 150 μg de N par souris. Mais ces chiffres ne peuvent préjuger du seuil d'activité de ces fractions solubles.

Abbréviations: ARN, acide ribonucléique; ADN, acide deoxyribonucléique.

L'électrophorèse permet donc d'obtenir des fractions solubles douées de pouvoir antigénique. Sauf dans un cas, la solubilisation du matériel antigénique de départ n'a toutefois été que partielle. L'analyse chimique des fractions solubles actives est superposable à celle des fractions obtenues par chromatographie sur phosphate calcique par OTH ET CASTERMANS[3]. Ces faits suggèrent que le pouvoir antigénique pourrait être supporté par une glycoprotéine, dont la forte charge positive expliquerait les caractères particuliers de solubilité et peut être aussi l'affinité pour l'ARN (ou l'ADN[7] dans certains cas).

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Elimination by the urine of orotic acid and orotidine in man after application of 6-azauracil

The principal pathway of biosynthesis of the pyrimidine components of nucleic acids can be characterized by the sequence of the intermediates:

aspartic acid → ureidosuccinic acid → dihydroorotic acid →
→ orotic acid → orotidylic acid → uridylic acid,

the key positions being occupied by derivatives of orotic acid[1,2]. Their isolation from biological material, however, is quite complicated. Orotic acid[3] and later even dephosphorylated orotidylic acid, orotidine[4], have been isolated only from some mutants of *Neurospora crassa*, deficient with respect to decarboxylase of orotidylic acid. The older detection of orotic acid in milk can be considered nowadays rather as a curiosity[5].

New possibilities were provided by the application of 6-azauracil which—as was found subsequently—is transformed to 6-azauracilriboside 5′-phosphate[6,7] and as a such inhibits the decarboxylase of orotidylic acid[8]. Inhibition of *Escherichia coli* cultures by 6-azauracil resulted in an accumulation of orotic acid[6] or—with some strains—of orotidylic acid[9] in the medium. At the same time, it was established that orotic acid and orotidine are eliminated en masse by the urine of normal and tumour-bearing mice treated with 6-azauracil or with its riboside[7,10], which served actually as the first direct proof of existence of a similar type of compounds in higher animals. Later, the compounds were identified even in acid extracts of tumours of animals which were treated with 6-azauracilriboside[11]. The present communication contains

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new information on the elimination by the urine of considerable amounts of orotic acid and orotidine by healthy subjects as well as by cancer patients, following application of 6-azauracil, which indicates that the derivatives of orotic acid occupy an important position in the biosynthesis of the pyrimidine components of nucleic acids even in man.

The urine of 4 patients suffering from stomach cancer and treated subcutaneously with 100 mg 6-azauracil/day and of a healthy man after a single dose orally of 100 mg of the analog was analysed. Pyrimidines, purines and their derivatives were adsorbed from acidified urine on active carbon, desorbed by aqueous alcohol–ammonia and analysed by descending paper chromatography in *n*-butanol–water. In order to achieve separation of substances with low R_F values the chromatograms were run for 48–72 h. The nucleic acid components were detected by photographing in u.v. light (Fig. 1). After elution of the individual compounds with 0.1 *N* HCl orotic acid was identified by its characteristic u.v.-absorption spectrum with a minimum at 243 mμ, a maximum at 280 mμ and the ratio of extinctions at 280 and 260 mμ equal to 1.78. Orotidine displayed a minimum at 234 mμ, a maximum at 267 mμ and the ratio of extinctions at 280 and 260 mμ equal to 0.76. After hydrolysis in 4 *N* H_2SO_4 the hydrolysate was shown to contain orotic acid with a typical absorption spectrum in the u.v. region. The ratio of nitrogenous base: pentose: phosphorus was found to be equal to 1.00:0.93:0.02.

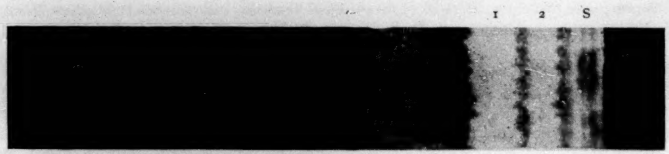

Fig. 1. Chromatogram of the orotic acid and orotidine isolated from the urine after application of 6-azauracil. Whatman No. 1 paper, developed by *n*-butanol saturated with water for 72 h, photographed by u.v. light. S, start; 1, orotic acid; 2, orotidine.

During the first 12 h after a single application of 100 mg 6-azauracil the healthy man eliminated approximately 75 mg orotic acid and 100 mg orotidine in the urine; later on, the two compounds could not be detected in the urine.

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Non-enzymic transamination between glycine and glyoxylate

Non-enzymic transamination reactions of the type

$$R'CH(NH_2)CO_2H + RCOCO_2H \rightleftharpoons R'COCO_2H + RCH(NH_2)\,CO_2H$$

under physiological conditions have been reported by NAKADA AND WEINHOUSE[1] for limited cases where R = H. The equilibria of the reactions were completely in favour of glycine formation. No evidence was obtained for non-enzymic transamination in the case where R and R′ are alkyl derivatives.

TABLE I

NON-ENZYMIC TRANSAMINATION BETWEEN [2-^{14}C]GLYCINE AND GLYOXYLATE

Additions	*Glyoxylate activity (counts/min/μmole)*				
	0 h	*3 h*	*5 h*	*28 h*	*98 h*
—	1.510	1,580	1,720	3.470	13.500
Cu^{++}	1,510	15,900	22,100	58,300	101,000
EDTA	1,510	1,490	1,570	1,870	2,220

[2-^{14}C]Glycine (0.005 *M*) (1.25 μC/)mole) and sodium glyoxylate (0.005 *M*) were incubated in Sørensen's 0.067 *M* phosphate buffer, pH 7, at 37°. Additions of $CuSO_4$ (0.5 m*M*) and ethylenediaminetetraacetate (EDTA) (5 m*M*) were made as indicated. Glyoxylate was isolated as the 2,4-dinitrophenylhydrazone and further purified by paper chromatography in *n*-butanol–0.3 % NH_3 (R_F *trans* isomer, 0.24; *cis* isomer, 0.36). Glyoxylate 2,4-dinitrophenylhydrazone (*trans* isomer) was counted at infinite thinness and estimated spectrophotometrically at 367 mμ in 0.5 % $NaHCO_3$.

The results presented in Tables I and II demonstrate a facile non-enzymic transamination between [^{14}C]glycine and glyoxylate at pH 7 and 37°. The reaction is apparently dependent on co-ordination-complex formation as indicated by the Cu^{++} catalysis and the inhibition by ethylenediaminetetraacetic acid. The reaction is also catalysed by OH^{-} and abolished at pH's lower than 6. The effect of the added Cu^{++} is reversed in the presence of pyridoxal phosphate (Table II). A comparison of the glycine–glyoxylate reaction with the homologous alanine–pyruvate system is shown in Table II.

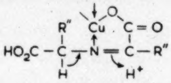
TABLE II

COMPARISON OF THE NON-ENZYMIC TRANSAMINATION BETWEEN (a) [1-^{14}C]GLYCINE AND GLYOXYLATE AND (b) [1-^{14}C]ALANINE AND PYRUVATE

System	*Additions*	*Keto acids counts at 24 h as % of total counts*
Glycine–glyoxylate	—	11.3
	Cu^{++}	30.6
	Cu^{++} + $PyrPO_4$	9.7
Alanine–pyruvate	—	< 1
	Cu^{++}	< 1
	Cu^{++} + $PyrPO_4$	77.7

[1-^{14}C]Amino acid (0.01 *M*, 0.8 μC/μmole) and keto acid (0.05 *M*) were incubated in Sørensen's 0.067 *M* phosphate buffer, pH 7, at 37°. Additions of $CuSO_4$ (1 m*M*) and pyridoxal phosphate ($PyrPO_4$) (10 m*M*) were made as indicated. Keto acid 2,4-dinitrophenylhydrazones were isolated, purified and counted as described in Table I.

The mechanism of the glycine–glyoxylate non-enzymic transamination may be envisaged as involving the co-ordination complex of a Schiff base, (I, R″ = H), which undergoes prototropic rearrangement:



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The facile transamination noted in the glycine–glyoxylate system as compared with the alanine–pyruvate system (Table II) would appear to be related to the absence of an α-alkyl substituent which through electron-release effects would inhibit prototropic rearrangement.

The effect of pyridoxal phosphate on the catalysis of the glycine–glyoxylate system by added Cu^{++} would appear from spectrophotometric evidence to be due to the formation of a pyridoxylideneglycine–copper complex of the type proposed by METZLER *et al.*[2] In view of the lack of significant participation of glycine in the pyridoxal-catalysed reactions described by METZLER AND SNELL[3], the formation of such a pyridoxylideneglycine–copper complex would effectively remove Cu^{++} from solution and so inhibit the transamination. The pronounced effect (Table II) of the α-alkyl substituent on the amino acid–pyridoxal phosphate–Cu^{++} reaction is in a direction opposed to its electron-release effect on the prototropic pyridoxylidene–glycine–azomethine structure. The effect of the α-alkyl substituent may here be envisaged as an influence on the free-energy change of the reaction exerted through hyperconjugative and inductive interaction with the carbonyl group of the keto acid product of the reaction. This formulation of the α-alkyl–substituent effect satisfactorily explains the observations of Table II and of METZLER AND SNELL[3], who have reported that the effect of an α-substituent in shifting the equilibrium of their amino acid–pyridoxal–Cu^{++} reaction towards keto acid and pyridoxamine formation is in the order

$$CH_3\text{–},\ CH_3CH_2CH_2\text{–},\ CH_3CH(CH_3)CH_2\text{–} > CH_3CH_2CH(CH_3)\text{—} > CH_3CH(CH_3)\text{—} \gg H$$

NAKADA AND WEINHOUSE[4] have reported on the basis of experiments employing an isotope-trapping technique that glyoxylate is an intermediate in the production of [^{14}C]formate from [^{14}C]glycine by rat-liver homogenates. It should be appreciated that the results of such experiments must be regarded as somewhat equivocal in view of the facile non-enzymic transamination reaction between glycine and glyoxylate described in this communication.

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The synthesis of glycogen in yeast

Enzymes catalysing the synthesis of glycogen from UDPG have been found in animal tissues[1–3]. A similar enzyme has now been purified about 500-fold from baker's-yeast extracts. The purification procedure involves the following steps: (a) grinding of the lyophilized yeast and subsequent extraction with 0.05 *M* Tris-maleate buffer, pH 7.5, followed by dialysis; at this stage, part of the enzyme is sedimentable at 20,000 × *g*; (b) treatment with 1 % digitonin, which brings most of the enzyme into solution; (c) immediate precipitation with satd. $(NH_4)_2SO_4$, and dialysis; (d) adsorption on retrograded amylose[4], and (e) elution from amylose with a conc. (100 mg/ml) glycogen solution, buffered at pH 7.5. Practically all the trehalose-synthesizing enzyme[5] remains in the supernatant, in the amylose-adsorption step.

No phosphorylase or amylase could be detected in the purified preparation under the usual assay conditions for these enzymes. A slight amylase activity was, however, observable in the presence of Ca^{++}.

Glycogen is required for activity, as shown in Table I. Since the enzyme used in this experiment was adsorbed on retrograded amylose, it would seem that the latter does not function as primer.

When [^{14}C]UDPG was used as substrate, the amount of radioactivity incorporated into glycogen was equivalent to the UDP formed, as can be seen in Table II.

TABLE I

GLYCOGEN REQUIREMENT FOR ACTIVITY OF THE ENZYME

The complete system contained 0.35 µmole UDPG, 1 µmole G-6-P, 2.4 mg glycogen, 10 µmoles Tris-maleate buffer, pH 7.5, 1 µmole EDTA and 0.01 ml of amylose suspension containing adsorbed enzyme (step d), in a total volume of 0.1 ml. After incubating for 30 min at 37° the UDP formed was determined as described by LELOIR AND GOLDEMBERG[1].

Incubation mixture	*UDP formed (mµmoles)*
Complete system	61
Complete system *minus* glycogen	6
Complete system *minus* glycogen and G-6-P	1

TABLE II

STOICHIOMETRY OF THE REACTION

The incubation mixture contained 0.5 µmole [^{14}C]UDPG (uniformly labeled in the glucose moiety, specific activity 17,200 counts/min/µmole), 1 µmole G-6-P, 10 µmoles Tris-maleate buffer, pH 7.5, 1 µmole EDTA and 0.02 ml of purified enzyme (step e) containing 2 mg of glycogen, in a total volume of 0.1 ml. After 90 min incubation at 30° UDP was determined according to LELOIR AND GOLDEMBERG[1]. Glycogen was isolated and counted as described by LELOIR *et al.*[2].

Incubation mixture	*UDP formed (µmoles)*	*Glucose incorporated into glycogen*	
		(counts/min)	*(µmoles)*
Complete system	0.20	3140	0.18
Complete system *minus* G-6-P	0.14	2300	0.13
Complete system, UDPG added at the end of incubation	—	20	—

Abbreviations: UDPG, uridine diphosphate glucose; UDP, uridine diphosphate; G-6-P, glucose 6-phosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate.

After treatment of the labeled glycogen with β amylase, the radioactivity was recovered in the liberated maltose.

While G-6-P increased the activity of yeast glycogen synthetase, the stimulation obtained was usually less than 2-fold (see Table II) as compared with 15- to 20-fold in the case of the animal enzymes[1,2].

A full report of these findings will be published elsewhere.

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The identification of lysolecithin in lipid extracts of brain

The presence of lysolecithin in lipid extracts of a number of mammalian tissues has recently been reported[1–5]. Its presence in extracts of brain has not, as far as we are aware, been described, although PIGHINI[6] in 1932 had obtained an alcohol-soluble, ether-insoluble substance from human brain which, both from its solubility properties and from certain of its biological actions, appeared to resemble a lysophosphatide. It was decided therefore to re-investigate PIGHINI's work, using more modern chromatographic methods for the separation of lysolecithin. Initial experiments showed that lipid extracts of normal human brain, prepared by PIGHINI's method, contained a haemolytic substance having the same R_F value as lysolecithin. We therefore next decided to extract the brain lipids by the method of FOLCH *et al.*[7] and to characterise this haemolytic substance more precisely.

Human brains were obtained post-mortem as soon as possible after death from cases in which there had been no clinical evidence of nervous disorder. Meninges were removed and the surface of the brain washed free from blood in gently running tap-water. Cerebral cortical grey matter was then dissected off and extracted. In addition, a number of scattered plaques were dissected from the unfixed brain of a case of multiple sclerosis.

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One specimen of a normal human frontal pole was obtained post-operatively, immediately placed in a vacuum flask at 0°, brought to the laboratory and treated as above. Whole rat brains were also used, and extracted immediately after killing by decapitation.

The choline-containing phosphatide fractions were separated from the total lipid extracts[7] on Al_2O_3 columns and then applied to silicic acid-impregnated papers[8]. Chromatograms were developed with $CHCl_3$–CH_3OH (4:1, v/v). Egg lysolecithin[9] was used as a marker.

Selected lanes were cut from each paper, stained with phosphomolybdic acid, washed and reduced with $SnCl_2$[10]. Areas corresponding to the lysolecithin spots were cut out from unstained lanes and eluted with CH_3OH–$CHCl_3$ (5:1, v/v). The eluates were evaporated in a rotary evaporator at 40° and dissolved for analysis in a known volume of $CHCl_3$.

Total P and P in the alkali-labile phospholipids (following hydrolysis by the method of SCHMIDT *et al.*[11]) was determined as described previously[12]. Fatty acids were determined titrimetrically after hydrolysis of lipid samples for 2 h in 2 N NaOH, followed by acidification and extraction of the fatty acids with ether. Choline was estimated by the method of WEBSTER[13], and haemolytic activity tested by the procedure of COLLIER[14].

Paper chromatograms of the choline phosphatides obtained from human brain have regularly shown the presence of 3 spots staining with phosphomolybdic acid, the slowest of which has an R_F resembling that of lysolecithin. Elution of this slowest spot from unstained papers, followed by re-chromatography on paper, showed the presence of a single spot with R_F corresponding to that of lysolecithin (Fig. 1).

Fig. 1. Chromatogram of human brain lysolecithin. A = Brain lysolecithin. B = Brain lysolecithin + added egg lysolecithin. C = Egg lysolecithin.

After elution from unstained papers this substance was in each experiment found to be haemolytic and to give total P:alkali-labile lipid P:choline:fatty acid ratios close to 1:1:1:1 (Table I). These data indicate to us therefore that total lipid extracts of brain contain appreciable amounts of lysolecithin. Confirmatory evidence of a preliminary nature has been obtained by infrared spectroscopy kindly carried out by Dr. W. L. G. GENT. It is possible of course that the lysolecithin in these extracts may have been formed in the brain post-mortem, or during the chemical manipulations involved in its isolation. It has, however, been isolated from the post-operative specimen of human brain in amounts comparable to those present in the post-mortem material, and is also present in rat brain extracted within 3 min of killing. It has also been shown that storage of the total lipid extract at −10° for 48 h causes no apparent increase in the size of the lysolecithin spot; furthermore, when lecithin was taken through the entire procedure of extraction and chromato-

TABLE I

ANALYTICAL DATA ON THE ELUATES

Tissue	μmoles/ml eluate solution				Molar ratios (A: B: C: D)
	A Total P	B Alkali-labile lipid P	C Choline	D Fatty acids	
Rat brain	0.31	0.29	0.30	—	1 : 0.93 : 0.98 : —
Rat brain	4.57	4.43	4.57	4.56	1 : 0.97 : 1.00 : 1.00
Rat brain	7.06	7.48	7.01	6.65	1 : 1.06 : 0.99 : 0.94
Post-operative human brain	0.47	0.41	0.48	—	1 : 0.87 : 1.02 : —
Post-mortem human brain	7.10	6.80	6.00	6.40	1 : 0.96 : 0.85 : 0.90
Post-mortem human brain	1.44	1.44	1.51	1.52	1 : 1.00 : 1.05 : 1.06
Post-mortem human brain	1.60	1.52	1.62	1.70	1 : 0.95 : 1.01 : 1.06
Post-mortem human brain	0.86	0.90	0.88	0.93	1 : 1.05 : 1.02 : 1.08
Post-mortem human brain	1.51	1.54	1.57	1.61	1 : 1.02 : 1.04 : 1.07
Plaque tissue from brain of case of multiple sclerosis	0.89	0.92	0.90	—	1 : 1.03 : 1.01 : —

graphic separation used in the brain experiments no evidence of lysolecithin formation was observed.

Although our methods are not yet entirely suitable for quantitative work (and for this reason the data in Table I are expressed as μmole/ml eluate solution), the amounts of lysolecithin present would appear to correspond to 1–4 μmoles/g fresh tissue. Further work is now in progress to put these observations on a quantitative basis.

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DIFFUSION OSMOTIC EQUILIBRIUM

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SUMMARY

A new method for measuring diffusion constants is described. In this method an equilibrium is established in a cell between upward diffusion and downward migration on to a semi-permeable membrane across which is established a strong osmotic gradient.

The diffusion constant of bovine serum albumin determined by this method agrees well with the value obtained with the boundary spreading method. The method can also be used for testing the homogeneity of colloidally dispersed substances.

INTRODUCTION

The method most commonly used for the determination of the diffusion constant of a substance is to establish a sharp interface between the solution of the substance and its solvent, and then to measure the spreading rate of the boundary. These measurements are made by optical means such as light absorption, light refraction or interferometry. Apart from the determination of diffusion constants, the boundary spreading method has also been used for testing the homogeneity of dispersions qualitatively. This was not always successful especially when mixtures of substances whose diffusion constants did not differ greatly, were examined. The diffusion constant of a substance is proportional to the square of the average distance traversed by a molecule from a given point in a given time interval. It follows, therefore, that although diffusion constants of the components in a mixture of two substances may differ markedly, the average distances traversed by the molecules of the individual components will necessarily differ less.

In this report a new approach is made to the determination of the diffusion constant whereby the quadratic distance term is resolved into two factors, a velocity and a linear distance component.

By this means it is possible to indicate inhomogeneity in colloidally dispersed systems when the conventional boundary spreading techniques give less satisfactory answers. The method is based on the establishment of equilibrium between downward migration of a substance on to a semi-permeable membrane and upward migration through the process of diffusion.

Abbreviations: P.E.G., polyethylene glycol; D.O.E., diffusion osmosis equilibrium; B.S.A., bovine serum albumin.

Theoretical

Consider a solution of a substance in a vertical cell, one end of which is closed by a semi-permeable membrane. Let the solution be in ionic equilibrium with an electrolyte solution above it and below the semi-permeable membrane. Further, let the substance migrate on to the membrane at a constant rate of V cm/sec and diffuse outwards at a rate of D cm^2/sec. Let C be the concentration at any level x cm removed from the membrane surface. When equilibrium is reached between outward diffusion and downward migration, the amount of substance which passes downwards through a definite level, equals that amount which passes upwards through that level. The amount of material which passes downwards through a section of unit area in time dt equals $V c \mathrm{d}t$, and the amount which diffuses outwards equals

$$-D\frac{\mathrm{d}c}{\mathrm{d}x}\cdot \mathrm{d}t$$

at equilibrium

$$Vc\mathrm{d}t + D\frac{\mathrm{d}c}{\mathrm{d}x}\cdot \mathrm{d}t = 0$$

Rearranging and integrating between the limits x_1 and x_2 we have:

$$V\,(x_1 - x_2) = D \ln\frac{c_2}{c_1}$$

from which follows

$$D = \frac{V\,(x_1 - x_2)}{\ln\dfrac{c_2}{c_1}} \qquad (1)$$

By establishing the concentration distribution in the column after the equilibrium state had been reached, the diffusion constant may be calculated.

Methods and apparatus

In Fig. 1 is a sketch of the apparatus used in the present work. A is the central section of the Tiselius electrophoresis cell, B is a container provided with two slots which correspond to the openings in the limbs of the electrophoresis cell. A cellophane membrane m is interposed between A and B. D is a levelling section provided with four slots, each of which has approximately three times the capacity of a rectangular cavity in A. Cavities 1 and 1′ are joined on to glass capillaries of 2 mm bore. Cavities 2 and 2′ are used for introducing the material into the cavities in A. The surfaces between D and A, and A and B are well greased and the cellophane membrane is held tightly between A and B by metal spring blades. The sections D and B are made of perspex.

Several methods were tried to produce a constant rate of migration of a colloid suspension on to a semi-permeable membrane, but without success. A positive pressure on the column caused leaks to occur in the apparatus. Movement of the dispersed phase by the application of an electrical potential gradient across the column resulted in the accumulation of electrolyte on the membrane, an effect observed previously during electrodecantation of proteins[1]. Strong osmotic gradients that were created across the semi-permeable membrane at the bottom of the cell caused the desired migration of the fluid on to the membrane. By the use of suitable substances such as P.E.G., any desired osmotic gradient may be obtained.

The material to be investigated was dialysed overnight against a suitable concentration of P.E.G. dissolved in saline, before dilution in saline to the required concentration. The container B was filled with the P.E.G. solution and all the air trapped in the slots was removed by inversion of the apparatus. The solution to be investigated was introduced into the two limbs of A *via* cavities 2 and 2′. The cavities 1 and 1′ were filled with saline. Section D was moved across so that 1 and 1′ were in apposition with the cavities in A. The interfaces which formed between solution and solvent were lowered to approx. 1 cm from the membrane's surfaces with the aid of a finely drawn pipette, following the technique of Polson[2] and Kahn and Polson[3]. To ensure against leakages, cavities 2 and 2′ were filled with molten vacuum grease, and the glass capillaries were attached to 1 and 1′ and filled with saline. The apparatus was attached to a frame and lowered into the electrophoresis bath which was regulated at room temperature[*]. After temperature equilibrium had been obtained, the heights of the menisci were carefully noted.

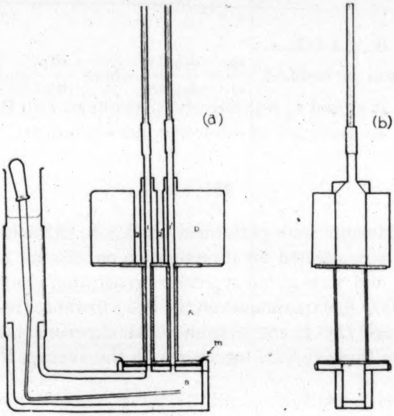

Fig. 1. (a) Sectional elevation of apparatus in which D.O.E. experiments were performed. A, central chamber of Tiselius electrophoresis apparatus. B, P.E.G. container provided with a Pasteur pipette and plastic tube to agitate the fluid at intervals. D, levelling section with 4 slots, 1, 1′, 2 and 2′. 1 and 1′ are joined on to capillary tubes. Fig. 1. (b) Side view of apparatus.

The concentration gradients in the cells were recorded daily with the Lamm scale method[4], and the lowering of the saline menisci in the capillaries noted. After each observation the capillaries were filled to their original levels. Photographic recordings of the concentration gradients in the cells were made daily until the state of equilibrium had been reached.

To convert the dimensions on the photograph, as plotted on the graph paper, to those in the diffusion cell, the abscissae were multiplied by a conversion factor—

$$K = \frac{l - b}{l} \cdot \frac{1}{G}$$

---

[*] No disturbance sufficiently large to influence the course of the experiment was noticed when this procedure was followed.

where l and b are the optical distances from the scale to the camera lens and from the scale to the centre of the diffusion cell respectively. G is the photographic enlargement factor[4]. Equation (1) now becomes:

$$D = \frac{V\,(x_1 - x_2)}{\ln \frac{c_2}{c_1}} \cdot K$$

In the present work $K = 1.111$.

When equilibrium is reached $\frac{c_2}{c_1} = \frac{dc_2/dx}{dc_1/dx}$ where $\frac{dc_2}{dx}$ and $\frac{dc^1}{dx}$ are the concentration gradients at x_2 and x_1 respectively, (SVEDBERG AND PEDERSEN[5]). The concentration gradients are recorded as refractive index gradients.

RESULTS

Several D.O.E. experiments were performed on B.S.A. (Armour) and rabbit serum. The latter substance was selected for investigation on account of its inhomogeneous character. In Figs. 2 and 3 are given typical concentration gradient curves obtained during the course of D.O.E. experiments on the two substances respectively. In Figs. 4 and 5 are given the static D.O.E. curves from similar experiments on B.S.A. and rabbit serum. The data from these curves, together with the average V values of Table III

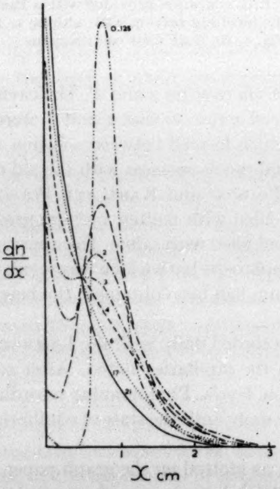

Fig. 2. Curves obtained during a D.O.E. experiment on B.S.A. (Armour). The numbers denote the time interval in days from the start of the run. Equilibrium reached in 13 to 17 days. The ordinates are the refractive index gradients. ———, equilibrium curve.

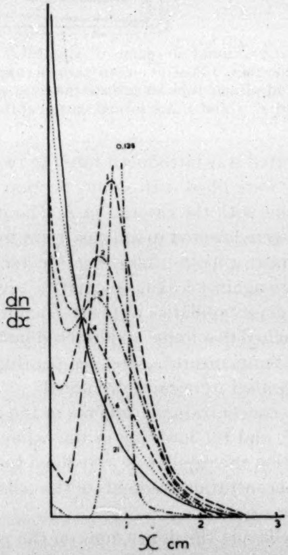

Fig. 3. Curves obtained during a D.O.E. experiment on rabbit serum performed concurrently with the B.S.A. run (Fig. 2). The numbers denote the time intervals in days. Equilibrium was reached after approx. 21 days. The ordinates are the refractive index gradients. ———, equilibrium curve.

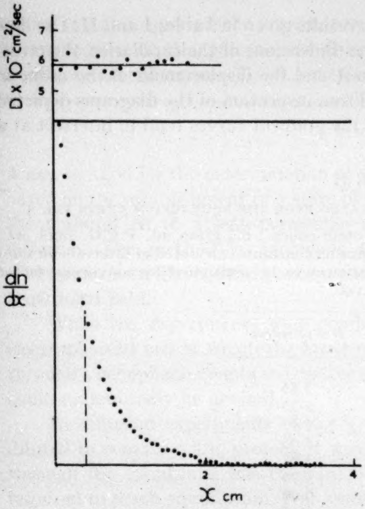

Fig. 4. Equilibrium curve obtained during a D.O.E. experiment on B.S.A. The dashed line is the ordinate at the final point of intersection of the gradient curves used as reference ordinate C_2. The straight line at top of diagram shows the variation of D with x.

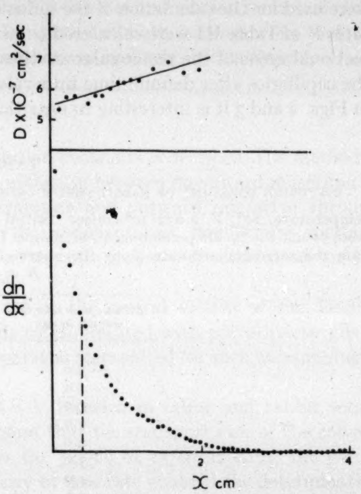

Fig. 5. Equilibrium curve obtained during a D.O.E. experiment on rabbit serum. The dashed line is the ordinate at the final point of intersection of the gradient curves used as reference ordinate C_2. Straight line at top of diagram shows the variation of D with x.

TABLE I

DIFFUSION CONSTANT OF B.S.A. CALCULATED FROM THE EQUILIBRIUM CURVE FIG. (2)

Temperature, 20°; V, $1.15 \cdot 10^{-6}$ cm/sec. Initial protein concentration 0.5 g/100 ml. P.E.G. (mol. wt. 6000) concn. 8.0 %. Dispersion medium: saline. Diffusion constants calculated at intervals (in mm) from the reference ordinate along the curve. Distances to be multiplied by conversion factor $K = 1.111$.

Distance from reference ordinate in mm	$D \times 10^7$ *cm²/sec*
—3.0	5.70
—2.0	6.28
—1.0	5.74
1.0	5.80
2.0	6.16
3.0	5.67
4.0	5.6
5.0	5.74
6.0	6.05
7.0	5.54
8.0	5.72
9.0	5.91
10.0	5.98
11.0	6.00
12.0	6.04
13.0	
14.0	5.91
Average	5.87
Corrected =	5.96

were used for the calculation of the diffusion results given in Tables I and II. The flow rates V of Table III were calculated from the dimensions of the capillaries, the cross-sectional areas of the rectangular cavities in A and the displacement of the saline in the capillaries after definite time intervals. From inspection of the diagrams depicted in Figs. 2 and 3 it is interesting to note that the gradient curves tend to intersect at a

TABLE II

DIFFUSION CONSTANT OF RABBIT SERUM CALCULATED FROM THE EQUILIBRIUM CURVE FIG. 3

Temperature, 20°; V, 0.972 · 10^6 cm/sec. Initial protein concn., 0.5 g/100 ml. P.E.G. (mol. wt., 6000) concn. 8.0 %. Dispersion medium: saline. Diffusion constants calculated at intervals (in mm) from the reference ordinate along the curve. Distances to be multiplied by conversion factor $K = 1.111$.

Distance from reference ordinate in mm	$D \times 10^7$ *cm²/sec*
—3.0	5.35
—2.0	5.75
—1.0	5.87
1.0	5.55
2.0	5.82
3.0	5.79
4.0	6.06
5.0	6.26
6.0	6.27
7.0	6.37
8.0	6.33
9.0	
10.0	6.71
11.0	
12.0	6.95
13.0	
14.0	6.58
15.0	
16.0	6.69

TABLE III

FLOW RATES OF THE SALINE THROUGH THE CELLOPHANE MEMBRANE FOR THE LIMBS CONTAINING B.S.A. AND RABBIT SERUM RESPECTIVELY

Capillary diameter 0.2037 cm. Cross. area of cells 0.790 cn^2.

Time interval in hours	*V in B.S.A. cell*	*V in serum cell*
47.75	1.201 · 10^{-6} cm/sec	1.006 · 10^{-6} cm/sec
28.00	1.147 · 10^{-6} cm/sec	0.961 · 10^{-6} cm/sec
24.5	1.111 · 10^{-6} cm/sec	0.932 · 10^{-6} cm/sec
23.5	1.167 · 10^{-6} cm/sec	0.966 · 10^{-6} cm/sec
25.41	1.206 · 10^{-6} cm/sec	0.978 · 10^{-6} cm/sec
20.00	1.147 · 10^{-6} cm/sec	1.023 · 10^{-6} cm/sec
22.66	1.162 · 10^{-6} cm/sec	0.978 · 10^{-6} cm/sec
28.66	1.158 · 10^{-6} cm/sec	0.961 · 10^{-6} cm/sec
26.00	1.090 · 10^{-6} cm/sec	0.921 · 10^{-6} cm/sec
24.5	1.114 · 10^{-6} cm/sec	0.932 · 10^{-6} cm/sec
	Average 1.150	Average 0.972 · 10^{-6} cm/sec

point. It is also significant to note that the final position of intersection is reached early in the course of the process and that the curves of both homogeneous and inhomogeneous substances (B.S.A. and Serum) behave similarly in this respect.

DISCUSSION

A new method for the determination of diffusion constants is decribed. The method is based on the establishment of a state of equilibrium between downward migration of the material on to a semi-permeable membrane and outward migration through diffusion. The method is analogous to the sedimentation equilibrium method, (SVEDBERG AND PEDERSEN[5]), except that an osmotic gradient is substituted for the centrifugal field.

While the experiments were conducted in the central section of the Tiselius electrophoresis cell of which the lower ends communicated with polyethylene glycol through a cellophane membrane, better apparatus more suited for such measurements could undoubtedly be devised.

In diffusion experiments on 0.5 % B.S.A. solution in saline and rabbit serum diluted to contain 0.5 % protein, it was found that the transport rate of the solvent through the membrane was constant over the period of 15 to 21 days, the period required to reach equilibrium. This constancy of flow rate enabled the determination of a reliable diffusion constant. The diffusion constant of B.S.A. determined from the equilibrium curve, *i.e.* $5.95 \cdot 10^{-7}$ cm^2/sec is in good agreement with the value $6.15 \cdot 10^{-7}$ cm^2/sec obtained for human serum albumin using the boundary spreading technique combined with the LAMM scale method[6]. The diffusion constants calculated from the equilibrium curve of rabbit serum showed an increase with distance from the membrane surface. This is to be expected, as serum is known to be a mixture of protein components of different molecular weights.

The fluctuations in the values of the diffusion constants can be ascribed to inaccuracies in the scale line measurements. These errors could, most probably, be ruled out if the more accurate interferometric method of registering the concentration gradient be used.

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RIBONUCLEIC ACID AND THE FORMATION OF AMYLASE IN CELL FREE PREPARATIONS FROM PIGEON PANCREAS

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SUMMARY

1. The transfer of radioactivity from [^{14}C]precursor protein into amylase enzyme protein in a pancreas extract depends upon the presence of adenosinetriphosphate, arginine and threonine; it is abolished if ribonuclease is added.

2. The transformation of the precursor protein into amylase has been separated into two processes. In the first, a fraction of the RNA of the pancreas is changed into a specific RNA. This process requires ATP, arginine and threonine and is not inhibited by chloramphenicol. The second process, which is inhibited by chloramphenicol, requires only the specific RNA and a pancreas extract and leads to the formation of amylase.

3. The RNA which has this specific action is formed only from the RNA of the larger cytoplasmic granules (mitochondria etc.), the soluble and the microsomal RNA being inactive in this reaction.

INTRODUCTION

Discussion of the interconnection between RNA metabolism and the formation of secretory enzymes[1-3] has led to no conclusions, mainly because the formation of some proteins and the turnover of the total RNA* have been compared[3].

The first definite connection between RNA and protein synthesis was only recently established when the soluble RNA was recognized as being the primary acceptor of activated amino acid residues[4,5]. It is rather difficult to visualize the formation of the fine structure of a protein by supposing that all the information is already provided when the s-RNA–amino acid complexes are assembled in the correct order.

In our previous work we observed that amylase is formed in cell free preparations of pancreas and that then no incorporation of amino acids occurs. Such preparations lose their ability to form amylase after treatment with ribonuclease. According to our hypothesis, amylase is formed in these cell free systems from a protein precursor and, if this is true, it is to be expected that the process is essentially a change in the secondary or tertiary structure of the protein. The inactivating effect of ribonuclease is by itself not sufficient proof of the participitation of RNA. In the present experiments we have attempted to show in a more direct way that RNA plays a specific part in the transformation of precursor into amylase.

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* Abbreviations used: ATP, adenosinetriphosphate; RNA, ribonucleic acid; s-RNA, soluble RNA; g-RNA, RNA of the heavier cytoplasmic granules.

EXPERIMENTAL

Slices, mitochondrial fraction of pancreas, and acetone-dried powder from pigeon pancreas were prepared according to methods described earlier[6,7]. Enzyme activity was assayed by the method of SMITH AND ROE, slightly modified. Amylase was isolated from experimental mixtures by the method of STRAUB[8]. The radioactivity of the isolated and dialysed amylase was measured with a thin window counter, using very thin layers ($\leqslant 0.1$ mg/cm^2). Protein content of these samples was determined according to LOWRY *et al.*[9]. RNA was measured optically by the absorption of the solution at 260 mμ. The method of GIERER AND SCHRAMM[10] was used for the isolation of RNA, the only modification being that deproteinization was carried out in a glass homogenizer by homogenizing the tissue with 50 % phenol for 10 min at 0°. pH 5 enzyme was prepared from the 105.000 × *g* supernatant of a sucrose homogenate of pigeon liver, according to the method of HOAGLAND *et al.*[11].

RESULTS

Effect of ribonuclease on amylase formation and transfer of label from precursor protein to amylase

Previous experiments have shown that the amylase content of a mitochondrial fraction, or of a freshly prepared extract from acetone dried pancreas, is increased upon incubation with ATP, arginine and threonine[7]. As there is no incorporation of free amino acids during this process, amylase may be formed from a protein precursor. Indeed, if the mitochondrial fraction is obtained from pancreas slices previously incubated with radioactive glycine, then upon incubation of the mitochondrial fraction with ATP, arginine and threonine, the radioactive label of the amylase protein is substantially increased[12].

We find that this transfer of label from precursor protein to amylase is inhibited by ribonuclease treatment.

Pigeon pancreas slices were incubated aerobically in Krebs bicarbonate saline solution at 37° with [1-^{14}C]glycine (specific activity 7.5 μC/μM) using $1.7 \cdot 10^6$ counts/min/g slice wet weight, in presence of 0.2 % glucose and 0.2 % caseine hydrolysate. After 10 min the slices were rinsed with cold saline and rapidly dried with cold acetone. The dry powder was extracted with 4 volumes of distilled water and the extract was centrifuged at 0° for 10 min at 20,000 × *g*. To 1 ml of the supernatant were added 50 μmoles ATP, 5 μmoles L-arginine, 10 μmoles of DL-threonine and Krebs saline solution in a final volume of 2 ml. Some of the salts were dissolved in glycerol to obtain a glycerol concentration of 40 % in the experimental mixture. The mixture was divided into two 1 ml portions, one of them receiving in addition 10 μg of crystalline ribonuclease and both were incubated at 37° for 1 h. Samples were withdrawn every 15 min for the determination of amylase enzyme content and aliquots were used for the micro-isolation of amylase[8] at zero time and after 60 min incubation. The radioactivity of the purified samples was determined and the total label in amylase was calculated from the specific radioactivity and amylase enzyme activity of the experimental mixtures. Fig. 1 gives the result of an experiment which shows that the addition of ribonuclease inhibits both the increase in amylase enzyme activity and the transfer of radioactivity.

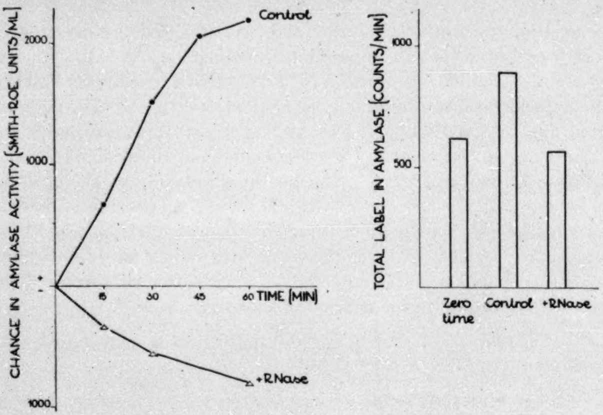


Fig. 1. Effect of ribonuclease on amylase formation and amylase labelling in a soluble system from pigeon pancreas pre-incubated with [1-^{14}C]glycine. Initial amylase activity: 9000 units/ml. Left: change in amylase content. Right: total label in amylase.

The role of "specific" RNA in the transformation of precursor protein into amylase

The fact that ribonuclease inhibits amylase formation in the soluble system[6] suggests that RNA plays some part in this process. We found the "mitochondrial fraction" to be the most adequate system for the study of this problem. It has the advantage that it is able to form relatively more amylase than the soluble system can. Like the "soluble system", the suspension of the heavier cytoplasmic granules does not incorporate free amino acids into the amylase protein, yet it is able to transfer the radioactivity from the labelled precursor into amylase, if ATP, arginine and threonine are present[12].

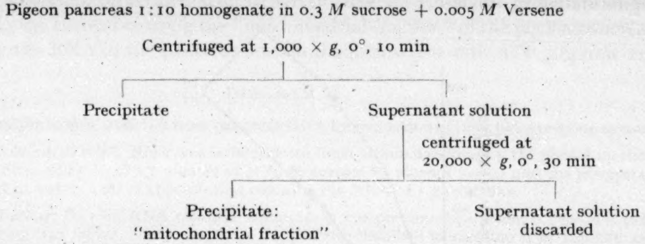
We assumed, as a working hypothesis, that the transformation of the precursor into amylase is preceded by some kind of alteration of the RNA, and that ATP, arginine and threonine are required to bring about this transformation. Using the "mitochondrial fraction", we were indeed able to separate the process into these two steps.

In these experiments we have used an experimental procedure which is summarized and described in detail in Diagram I. The procedure consists of two steps. First, the "mitochondrial fraction" is incubated with ATP, arginine and threonine. In presence of these ingredients, amylase would be formed in this system. However, in the present instance the incubation is carried out in presence of chloramphenicol, which inhibits the formation of amylase. After this incubation period, the RNA of the mixture is isolated and in Step 2. this RNA preparation is added to a freshly prepared "soluble system".

DIAGRAM I

Step 1. Formation of specific g-RNA in the "mitochondrial system"

a. Preparation of "mitochondrial system":



b. Incubation: "Mitochondrial fraction" obtained in the previous operation from 1 g pancreas suspended in Versene free 0.3 M sucrose, yielding about 1 ml of suspension. An equal volume of correspondingly concentrated Krebs' bicarbonate saline solution containing 50 µmoles ATP, 5 µmoles L-arginine and 10 µmoles DL-threonine added, + 0.1 mg/ml chloramphenicol. The mixture is incubated for 20 min at 37°.

c. Isolation of specific g-RNA. RNA was isolated from the reaction mixture of the previous operation by the phenol method of GIERER AND SCHRAMM[10]. After removing the phenol with ether and the ether with a stream of air, the RNA was precipitated with 60 % ethanol after addition of 0.1 volume of 20 % K-acetate. The precipitated RNA was dissolved in the minimum amount of water.

Step 2. Test for the effect of RNA on the formation of amylase.

a. Preparation of the "soluble system". Pigeon pancreas is rapidly homogenized twice successively with an excess of cold acetone and rapidly dried. The powder is extracted with 4 volumes of dist. water for 10 min, the mixture is centrifuged at 0° for 10 min at 20,000 × g. The precipitate is discarded, the supernatant solution is the "soluble system". It is always freshly prepared immediately before use.

b. Incubation. The RNA isolated in Step 1.c., is mixed with 0.5 ml of the soluble system and supplemented with Krebs' bicarbonate saline solution and glycerol, in order to give the correct isotonic salt concentration and a glycerol concentration of 40 %. Incubation is carried out for 45 min at 37° and 100 µl samples are withdrawn every 15 min for the determination of amylase enzyme content. These samples are immediately diluted 1 : 200 with ice-cold distilled water.

Such a "soluble system" alone gives no increase in amylase enzyme content upon incubation at 37° (Fig. 2, curve 4), but its amylase enzyme content increases if it is incubated in the presence of ATP, arginine and threonine (Fig. 2, curve 1). Fig. 2, curve 3, represents the result of an experiment in which the soluble system was incubated with the RNA isolated from the incubation mixture of a "mitochondrial fraction", as described in Diagram I, Step 1. It shows that this RNA preparation is able to replace the ATP, arginine and threonine. If this RNA preparation alone is added to the soluble system it leads to the formation of amylase. Fig. 2 curve 2 shows the result of a control experiment, in which the soluble system was supplemented with an RNA preparation, which went through the procedure described in Diagram I, except that the incubation mixture in Step 1 was not incubated at all, but the RNA was isolated immediately after mixing all the ingredients. It is evident that the RNA originally present in the "mitochondrial fraction" is inactive and that it acquires its specificity only upon incubation with ATP, arginine and threonine.

In the above experiment, we did not use all of the RNA of the pancreas, but only the fraction which is included in the heavier granules. This RNA of the heavier granules (g-RNA) can be transformed into a specific g-RNA, which takes part in the formation of amylase.

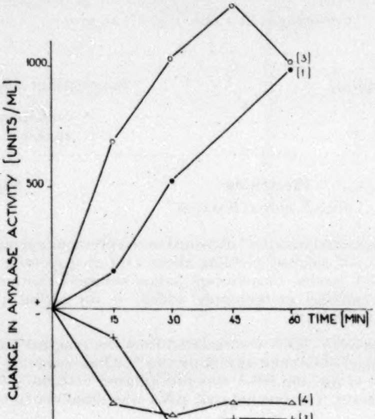


Fig. 2. Amylase formation in "soluble system", effect of specific g-RNA on amylase formation. The experimental technique is described in detail in Diagram I. 1: ATP, arginine and threonine added, 2: g-RNA, from non incubated sample added (about 5 mg), 3: "specific" g-RNA added (about 5 mg), 4: no addition. Initial amylase activity: 4000 units/ml.

It appears that this "specific g-RNA" is accumulated only in the presence of chloramphenicol. If the experiment is carried out as in Diagram I, but in Step i the incubation is carried out without chloramphenicol, the RNA isolated from such a mixture gives no effect in the soluble system. Similar negative results were obtained in experiments in which, in Step 1, the ATP, arginine or threonine were left out: the g-RNA isolated from such mixtures was always inactive in Step 2.

During the usual isolation of RNA by the phenol method, some of the ATP is precipitated along with the RNA. The question arises whether this ATP is perhaps needed for Step 2. In some experiments we have therefore washed the precipitated RNA with a slightly acid (pH 3) calcium chloride solution. The RNA is not dissolved in this solution, but all traces of ATP are removed by it. (This was controlled by precipitating and washing the RNA after addition of [^{32}P]ATP.) We were able to show that the carefully washed RNA, free of ATP, was active in Step 2.

Addition of ATP, arginine and threonine, together with specific RNA, in Step 2 did not substantially increase the amylase formation. The active RNA preparations have been shown to contain, after hydrolysis, only traces of these amino acids. Such traces do not give any amylase formation. It can therefore be concluded that in Step 2 it is the specific RNA which effects the transformation of precursor into amylase and that ATP, free arginine and threonine play no further part in Step 2, provided that enough specific RNA is added.

The next problem investigated was the nature of Step 1, namely, the reaction in which g-RNA is changed into the specific g-RNA. As this reaction requires the presence of ATP and two amino acids, the possibility exists that an amino acid activation process is somehow involved. To examine this possibility we have devised another experimental procedure, summarized in Diagram II. In this procedure Step 1 is much simpler. Instead of using the "mitochondrial fraction" of the pigeon pancreas, we use only the RNA of the pancreas and do the incubation with ATP, arginine and

DIAGRAM II

Step 1. Formation of specific RNA from pancreas RNA by the action of liver pH 5 enzyme system.

a. Preparation of RNA. RNA was isolated from fresh pigeon pancreas by the phenol method, precipitated after addition of 0.1 volume of 20% K-acetate by alcohol (60%) and the precipitate was dissolved in water. 1 ml of this solution contains the RNA of 1 g pancreas.

b. Incubation: To 1 ml RNA solution, obtained in the previous operation, 0.5 ml of a pH 5 enzyme solution was added, containing 5–10 mg protein dissolved in medium A of KELLER AND ZAMECNIK[13], plus 50 μmoles of ATP, 10 μmoles of DL-threonine, 5 μmoles of L-arginine and 0.1 mg/ml of chloramphenicol, final volume: 2 ml. The mixture is incubated for 20 min at 37°. The pH 5 enzyme preparation was obtained from pigeon liver, using the method of HOAGLAND[4].

c. Isolation of specific RNA. After incubation, the RNA of the experimental mixture was isolated again by the phenol method and, after precipitation with alcohol, it was dissolved in the minimum amount of water. This specific RNA was used in Step 2.

Step 2. Test for the effect of RNA on the formation of amylase. This is performed in exactly the same way as in Step 2 of Diagram I.

TABLE I

AMYLASE FORMATION IN "SOLUBLE SYSTEM" FROM PIGEON PANCREAS AFTER ADDITION OF "SPECIFIC" RNA OBTAINED THROUGH THE ACTION OF LIVER pH 5 ENZYME

The experimental technique is summarised in Diagram II. Step 1: the formation of the "specific" RNA. Step 2: amylase formation in the "soluble system". Results are expressed as the change in amylase enzyme content in Step 2, after the addition of the RNA formed in Step 1. In all the experiments recorded here, the zero time amylase content was between 4,500 and 5,800 units/ml.

Expt. No.	*Factor omitted (—) or added (+) Step 1*	*Added in Step 2*	*Change in amylase units/ml*
1 a	(Complete system)		+ 1000
b	— RNA		+ 150
c	— threonine, — arginine		— 50
d	— pH 5 enzyme		+ 150
e	— ATP		— 500
2 a	(Complete system)		+ 1080
b	— threonine, — arginine + serine + lysine + tryptophan + leucine + isoleucine + proline*		— 380
3 a	(Complete system)		+ 1150
b	— threonine		+ 500
c	— arginine		+ 400
d	— threonine — arginine + 16 other amino acids*		— 250
4 a	(Complete system)	No addition	+ 1800
b	(Complete system)	0.1 mg/ml chloramphenicol	— 200

* The concentration of amino acids other than arginine and threonine was in all cases 1 m*M*.

threonine in the presence of pH 5 enzyme from pigeon liver. After incubation, the RNA of the reaction mixture is isolated again and this RNA is tested in Step 2, its ability to form amylase in a "soluble system" being investigated.

The results of such experiments are summarized in Table I. It is seen that amylase is formed in the "soluble system" upon addition of the specific RNA which was obtained according to Step 1 of Diagram II. In other words, the specific g-RNA formed in pancreas "mitochondrial fraction" can be replaced by a specific RNA, obtained from pancreas RNA through the action of the pH 5 enzyme complex,—provided that ATP, arginine and threonine are present and that chloramphenicol is added. Arginine and threonine are necessary constituents in Step 1 and cannot be replaced by other amino acids (Table I).

Other experiments recorded in Table I show that the pancreas-RNA and ATP are absolutely necessary ingredients in Step 1. It should be remembered that the pH 5 enzyme from liver contains most of the s-RNA and this is naturally isolated from the incubation mixture of Step 1. But the negative result obtained in Expt. 1 of Table I* shows that this liver s-RNA does not acquire specificity as the pancreas RNA.

The last experiment in Table I gives the expected result: chloramphenicol, which does not inhibit Step 1, *i.e.* the formation of the specific RNA, inhibits the second step, *i.e.* the formation of amylase.

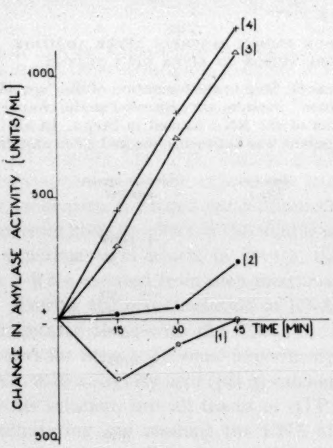


Fig. 3. Effect of chloramphenicol on the formation of "specific RNA". The experimental points indicate amylase formation in the soluble system after addition of different RNA preparations, which were obtained according to the experimental technique described in Diagram II, but using different concentrations of chloramphenicol in Step 1. Curve 1: effect of RNA obtained in the absence of chloramphenicol, Curves 2–4: effect of RNA obtained in the presence of 1 μg/ml, 10 μg/ml and 100 μg/ml of chloramphenicol respectively. The amount of RNA added was in all cases between 5–6 mg. Initial amylase activity: 5800 units/ml.

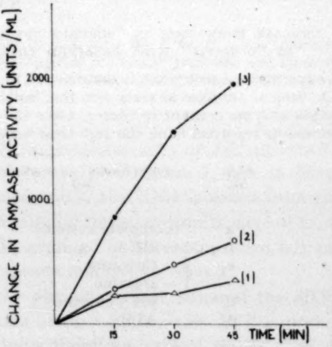


Fig. 4. Amylase formation after the addition of different amounts of "specific" RNA. The experimental technique is described in Diagram II. Additions to the soluble system from the same "specific" RNA preparation: 1: 0.85 mg, 2: 2.85 mg, 3: 8.5 mg "specific" RNA. Initial amylase activity: 6,500 units/ml.

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* It will be shown later (Fig. 5) that even the pancreas s-RNA fraction is not transformed into a specific RNA; this fraction is inactive in the amylase formation.

Fig. 3 shows an experiment, also performed according to Diagram II, in which the chloramphenicol concentration of the incubation mixture in Step 1 was varied. It is very interesting that chloramphenicol proves to be an essential component of the system in which the specific RNA is accumulated (Step 1), regardless of whether this is a mitochondrial fraction from pancreas, or a pH 5 enzyme preparation.

Fig. 4 gives the results of an experiment which was also performed according to the plan of Diagram II. In this instance, in Step 2, the amount of specific-RNA added to the "soluble system" was varied. It follows that the amount of RNA is still rate limiting. From these experiments it does not follow that the absolute amount of specific-RNA is identical with the amount of RNA added. We have yet no idea how much of the RNA is transformed into a specific RNA and, because our knowledge of the fractionation of RNA is rather rudimentary, we have not attempted to investigate this problem.

There is, however, an indication that not all of the pancreas RNA may be transformed into specific RNA. In the first type of experiments—those done according to Diagram I—we obtained positive results with an RNA which was formed within the "mitochondrial fraction" and this fraction contains less than half of the cytoplasmic RNA of the pancreas. It contains only traces of the microsomal and s-RNA. We have investigated the question whether the microsomal and s-RNA can also be transformed into specific RNA or not. To test this problem, we made the following experiment:

A sucrose homogenate of pigeon pancreas was prepared and, after discarding the sediment obtained after 10 min centrifugation at 1,000 × g, the homogenate was centrifuged for 30 min at 0° at 20,000 × g. The sediment is the "mitochondrial

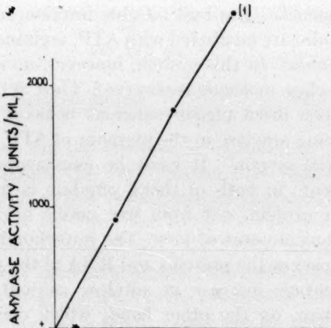

Fig. 5. Specific RNA formation from different RNA fractions of the pancreas. For a description of the experimental technique, see the text. The experimental points show the amylase content of the soluble system, after addition of pre-incubated RNA, 1: 4.6 mg RNA from the "mitochondrial fraction", 2: 6.2 mg from the microsome + s-RNA fraction. Initial amylase activity: 8,200 units/ml.

fraction" and the supernatant contains the microsomes and the soluble RNA. The supernatant was then acidified to pH 5.2 and the precipitate was collected, neutralized and dissolved. This contains most of the microsomes and the s-RNA, together with the acyl-activating enzymes.

The "mitochondrial fraction" was worked up according to Diagram I and the RNA isolated after Step 1 was tested in the soluble system. The effect of this g-RNA is shown in curve 1 of Fig. 5.

The pH 5 fraction, containing the microsomes the s-RNA and the amino acid-activating enzymes, was incubated with the usual concentration of ATP, arginine, threonine and chloramphenicol. In this instance neither RNA nor pH 5 enzyme was added. The latter was already present in sufficient quantity and the RNA in it was the RNA of the microsomes and the s-RNA. After 20 min of incubation the RNA of this mixture was isolated and tested in Step 2. As curve 2 of Fig. 5 shows, this RNA has no activity on amylase formation. The amount of RNA used in the two parallel experiments was of the same order of magnitude (see Fig. 5).

DISCUSSION

From our previous investigations[6,7,12] we concluded that the formation of amylase in the pancreas proceeds in several steps: in the intact cell a precursor protein is first formed *de novo* from amino acids and this in turn is transformed into the enzymically active amylase protein. The latter process may be regarded as the last phase of the formation of a specific protein. Whereas the formation of the amylase-precursor from amino acids occurs only in intact cells, the transformation of the precursor protein into amylase proceeds also in the isolated heavier cytoplasmic granules of a cell homogenate. The bulk of this fraction consists of mitochondria. When such isolated granules are incubated with ATP, arginine and threonine, amylase is formed from the precursor. In this system, however, no incorporation of arginine or threonine into the amylase molecule is observed. Their action is therefore indirect.

An extract of acetone dried pigeon pancreas behaves in a similar way. This "soluble system" is forming amylase in the presence of ATP, arginine and threonine, just as the "mitochondrial system". It must be emphasized that the two systems are in principle equivalent; in both of them, amylase is formed and it is formed only from the precursor protein, not from free amino acids. There are, however, differences from the technical point of view. The mitochondrial system is relatively simple: it contains only part of the proteins and RNA of the pancreas and it contains less amylase. The percentage increase in amylase content is therefore relatively higher. The soluble system, on the other hand, which contains nearly all of the soluble proteins, all of the RNA and of the amylase of pancreas, has the great advantage that it contains no permeability barriers. For this reason, in spite of the fact that the percentage increase of amylase in the soluble system is smaller than it is in the mitochondrial system, the soluble system can be used by itself for experiments in which the addition of ribonuclease, or the addition of polymeric RNA, are to be investigated. The soluble system has the disadvantage of containing already some ribonuclease, so that during incubation its RNA content is more rapidly depolymerised than is that of the mitochondrial system.

These technical considerations have led us to an experimental procedure in which the first phase of the transformation of the precursor protein into amylase is performed in a mitochondrial system and the specific RNA produced in this phase is tested in a soluble system. The use of chloramphenicol, which inhibits the second phase, makes it possible to separate these two steps.

We have already described circumstantial evidence suggesting that ribonucleic acid plays some part in the transformation of precursor protein into amylase. The present experiments provide the most direct evidence in favour of this assumption. In the system described above, the formation of amylase is dependent on the addition of a certain ribonucleic acid fraction and no formation of amylase occurs if other RNA fractions are added or if the RNA is omitted. It follows that the RNA involved in the process is a specific RNA. As Fig. 5 shows, if the cytoplasmic RNA of the pancreas is divided into two fractions, one of them containing the microsomal and soluble RNA, the other the RNA of the larger granules (g-RNA), it is only the latter fraction which leads to amylase formation. The complete inactivity of the microsomal and s-RNA in our system is particularly interesting in view of existing data, which show that these fractions play a very important part in the early phases of protein synthesis[4,5].

The specific g-RNA is not present in appreciable quantities in living pancreas tissue. But it is accumulated under specific conditions, if, in the presence of chloramphenicol, the heavier pancreas granules are incubated with arginine and threonine in the presence of ATP. The specific g-RNA is perhaps rapidly decomposed in the absence of chloramphenicol. Whether this is due to its being used up during its physiological function (formation of amylase), or by other (hydrolytic) action, or by both of these processes, cannot be yet decided.

According to the results described in this paper we may formulate the process of the transformation of precursor to amylase in the following way:

$$\text{g-RNA} \xrightarrow{\text{ATP, Thre, Arg}} \text{Specific g-RNA} \qquad (1)$$

$$\text{Precursor protein} \xrightarrow{\text{specific g-RNA}} \text{Amylase} \qquad (2)$$

The second reaction, but not the first, is inhibited by chloramphenicol. In view of the above considerations, the experimental data suggest the following picture. If we take a soluble system (or a mitochondrial preparation) and incubate it in the presence of ATP, arginine and threonine, then the two phases occur simultaneously. Specific g-RNA is constantly formed and used for the formation of amylase, because these systems contain the precursor protein, the g-RNA and the enzymes necessary for the formation of the specific g-RNA in the presence of ATP, arginine and threonine.

The nature of this specific g-RNA cannot yet be decided. Of great importance, however, is the fact that the formation of the specific RNA from g-RNA can be brought about, not only by the pancreas granules themselves, but also by the enzymes present in the pH 5 fraction from the liver, or by a purified fraction of amino acid activating enzymes*. The obvious implication of an RNA-amino acid complex has yet to be investigated.

When the precursor protein is transformed into the enzymically active amylase protein, no free amino acids are incorporated. Therefore the change effected by the specific g-RNA occurs in the secondary or tertiary structure of the protein. It is generally supposed that the specific structure of a protein is transmitted by the RNA, which directs the amino acid sequence of a protein, *i.e.* by the RNA of the s-RNA and microsome-RNA fraction. Our results emphasize the multiplicity of the information, the information about the secondary or tertiary structure being transmitted again by another RNA.

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* Unpublished observation of Dr. I. MILE and the authors.

It is hoped that the relatively simple change which leads from a precursor protein to an enzymically active protein is more useful for the study of the actual role that RNA may play as a template.

In all work on protein formation, the evaluation of the results becomes rather difficult if the possibility of a release must be considered. This is particularly true when, as in our investigation, the process studied occurs without the incorporation of free amino acids. PALADE AND SIEKEVITZ[14] have shown that amylase is released from incubated pancreas microsome granules and that this release is greatly increased by the addition of ATP. In our experience release of bound, preexisting amylase occurs in pancreas homogenates or in granular fractions of the pancreas. We have already summarized[7] our evidence, which suggested that such a release of bound enzyme and the formation of amylase from its precursor are two different processes which may be separated under definite experimental conditions. The results presented in this paper are taken as a decisive answer to this problem. In the experimental system devised by us, the increase in amylase enzyme activity is observed when neither ATP nor amino acid is present. In a positive and a negative experiment, the only difference deciding whether the amylase activity is increased or not, is the origin of the RNA added. We believe that in this way it is possible to distinguish between the ATP-induced release of amylase from microsomes[14] and the formation of amylase from a precursor protein observed in our experimental system.

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EFFECT OF X-RAYS ON NUCLEIC ACID ISOLATED FROM TOBACCO MOSAIC VIRUS

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SUMMARY

Breakage of RNA, X-irradiated after phenol extraction from tobacco mosaic virus, was measured using the ultracentrifuge equipped with u.v. absorption optics. The sedimentation patterns of control solutions of RNA show a homogeneous component corresponding in size to the fraction of monomer in the parent virus. By following the decrease of this RNA component with increasing X-ray dose, reproducible values for the 37 % survival dose, D_0, for breakage of the main chain can be obtained.

The indirect action of radiation was minimized by irradiating RNA in concentrated, oxygen-free, frozen solution. Under these conditions the D_0 for breakage was $21 \cdot 10^5$ R, while D_0 for dried RNA was $4.2 \cdot 10^5$ R, and for RNA in the virus was $4.0 \cdot 10^5$ R (see ref. 1). The anomaly could be due to intramolecular hydrogen bonding[2], since heating the irradiated RNA solutions to 65 or 70° drastically increased breakage leading to an effective D_0 of $4.4 \cdot 10^5$ R.

INTRODUCTION

The radiation sensitive volume of TMV, estimated from X-ray inactivation experiments, is much smaller than the particle size determined by other physical methods and has been shown to correspond to the RNA content of the virus[3,4]. Irradiation of TMV with doses of X-rays much larger than necessary to inactivate the virus does not produce any observable change in size and shape of the virus[5,7]. X-ray diffraction studies[6] indicate that the nucleic acid is embedded in the protein and thus is protected and held in a rigid configuration. When methods for the extraction of intact RNA from TMV became available, LAUFFER, TRKULA AND BUZZELL[7] showed that RNA isolated from irradiated virus was less viscous than that isolated from unirradiated virus. They suggested that both the viscosity decrease and the inactivation of the virus might be caused by radiation-induced breakage of the RNA strand. ENGLANDER, BUZZELL AND LAUFFER[1] developed a centrifuge technique to compare quantitatively the rate of breakage of the RNA isolated from irradiated TMV with the rate of loss of activity of the virus. However, it is possible that the breakage observed was the sum of the irradiation-caused damage plus additional breakage caused by the isolation

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Abbreviations: TMV, tobacco mosaic virus; RNA, ribonucleic acid.

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of RNA from the irradiated virus. The centrifuge technique of ENGLANDER *et al*[1]. has been used to study the breakage of RNA isolated before irradiation and to investigate the conditions which affect this breakage.

MATERIALS AND METHODS

Preparation of TMV

The type strain of TMV was isolated from systemically infected Turkish tobacco plants by the SIMMONS resin technique[8], a modification of an earlier low ionic strength method[9] which yields virus preparations containing a large majority of monomers[10]. The important features of this method are the extraction of the virus from the infected leaves at neutral pH in the presence of ion exchange resins and the purification of the virus by alternate low and high speed centrifugations at low ionic strength and neutral pH with the chelating agent, Versene. After purification the virus was stored at 5° in 10^{-3} M Versene solution until used. The virus can be stored in this way for long periods of time with no evidence of aggregation[9].

Extraction of RNA

The RNA was extracted from the preparations of TMV by the phenol method of SCHRAMM[11]. The method was modified slightly so that the virus was shaken for shorter periods of time with phenol: 4 min the first time and 1 min the second and third times.

Concentration determination

Concentrations of the various solutions were determined from measurement of of O.D. with a Beckman Model DU spectrophotometer. The RNA concentration was obtained by dividing the O.D. of the solution at 257 mμ by 253 $(\text{g}/100 \text{ ml})^{-1}$ (see ref. 12). The concentration of TMV solutions was determined by dividing the O.D. at 265 mμ by the extinction coefficient, 30.6 $(\text{g}/100 \text{ ml})^{-1}$ (see ref. 9). The concentrations of several TMV preparations were also checked by refractive index measurements made with a Phoenix differential refractometer. The value of $1.94 \cdot 10^{-4}$ $(\text{g/l})^{-1}$ was used for the specific refractive index increment of TMV in water at a wavelength of 436 mμ (see ref. 9).

Sedimentation

A Spinco Model E ultracentrifuge equipped with u.v. absorption optics was used for all centrifugations. Samples were contained in a standard four-degree, sector-shaped cell 12 mm deep. For centrifugation the RNA solutions were diluted to an O.D. at 257 mμ of 1.0, which corresponds to a concentration of 0.04 mg/ml. The nucleic acid was centrifuged at 59,780 rev./min in 0.02 M, pH 7.2 phosphate buffer. For TMV the centrifugations were done at a speed of 27,690 rev./min with a virus concentration of 0.3 mg/ml in 0.02 M phosphate buffer. All runs were made at about 4°. The rotor temperature, which was controlled by chamber refrigeration, drifted no more than a few tenths of a degree.

Pictures of the absorption pattern, taken every 4 min, were recorded on Kodak "commercial" film. To obtain sufficient film blackening, a 40-sec exposure was required. Films were developed with half strength Kodak DK-50 developer at 20° for exactly 2 min with continuous brushing. Photodensitometric tracings were made from the films with a modified Spinco Model RA Analytrol adjusted with calibrated neutral density filters to indicate the true O.D. of the film over a wide range. Only in a few cases were small corrections for non-linearity necessary.

Analysis of sedimentation data

The relative amount of homogeneous material in a solution was determined from tracings of the absorption pattern of the solution obtained during centrifugation (Fig. 1). These tracings show the changes, as material is sedimented, in the distribution of the solution density throughout the cell. There is a narrow region in which the tracings show an abrupt change in density, indicating a large number of particles moving at a uniform velocity. These particles can be considered to be a distinct component separate from additional inhomogeneous material. The amount of material in this component was calculated by comparing the change in this narrow region to the total difference in O.D. between the solution before and after the absorbing material is removed by centrifugation. The relative amount of homogeneous material was calculated from sedimentation patterns taken at three different times during the centrifugation. Each value was corrected for radial dilution[13] and the corrected amounts were then averaged.

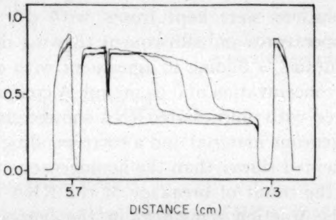

Fig. 1. Superimposed photodensitometric tracings of frames 1, 5, 7 and 9 of a typical preparation of unirradiated RNA.

For RNA solutions the apparent range of sedimentation coefficients calculated from the limits of this narrow region was 29–31 S. At least half of this spread can be attributed to diffusion and to the length of the exposure time; therefore this component can be considered homogeneous[14]. The average S_{20}^{w} value of 30 S for the homogeneous component agrees with that found by BOEDTKER[15] and by GIERER[16]. Both concluded the component consists of intact RNA particles of $2 \cdot 10^6$ molecular weight.

In TMV solutions 60 to 70 % of the material present was in a homogeneous fraction which had an average S_{20}^{w} of 190 S characteristic of TMV monomers[9, 17]. The RNA extracted from these virus preparations contained from 50 to 60 % of a homogeneous component; most of the rest of the material moved slower than the intact RNA and only about 10 % moved faster.

Irradiation procedure

The source of radiation was a Westinghouse deep-therapy X-ray machine with tungsten target and glass window. At 200 kV the output, calibrated with a Victoreen Roentgenmeter, Model 70, was 1900 R/min. The average effective wavelength of the radiation was 0.2 Å as determined by absorption in copper[3]. The solutions of RNA were kept frozen during irradiation. A long copper block holding the small sample cup was placed in a Dewar flask packed with dry ice. The sample temperature was —60° to —65° as measured with a copper-constantan thermocouple. The dry ice was replenished every 1–2 h during irradiation.

Measurements of infectivities

Infectivities of RNA solutions were measured by local lesion assays performed on *Nicotiana glutinosa* plants. Unknown samples were inoculated in parallel with standard samples at several dilutions in a Latin-square pattern to reduce variation resulting from plant or leaf position. Carborundum was dusted onto the leaves and 0.1 ml of the sample in 0.02 *M*, pH 7.2 phosphate buffer was pipetted onto the leaf. A clean spatula was used to spread the liquid over the surface of each leaf. The lesions were counted after five to seven days. Activities of the unknowns were expressed in terms of concentrations of standard sample necessary to produce an equal number of lesions.

RESULTS

Radiation-breakage of RNA

To prevent thermal denaturation of the RNA and to reduce the indirect effect of radiation, the RNA solutions were kept frozen with dry ice during irradiation. Freezing at dry ice temperatures and subsequent thawing did not affect the homogeneity of the RNA solutions, a finding in agreement with others[15,18]. Nucleic acid was first irradiated at a concentration of 0.04 mg/ml. A comparison of the sedimentation patterns of irradiated with unirradiated RNA showed that there was a decrease in the amount of homogeneous material and a corresponding increase in the amount of material which sedimented slower than the homogeneous fraction. This effect has been interpreted to be the result of breakage of the RNA strands[1]. In Fig. 2, the natural logarithm of the fraction remaining in the homogeneous component (ln survival) is plotted against the X-ray dose. The dose which leaves 37 % of the homogeneous component intact is called D_0 for breakage. The D_0 for breakage of frozen RNA irradiated at a concentration of 0.04 mg/ml was found to be $7.4 \cdot 10^5$ R. This is somewhat greater than the value of $4.0 \cdot 10^5$ R found by ENGLANDER *et al.*[1] for the D_0 of breakage for RNA extracted from irradiated virus.

Further investigation showed that both the presence of oxygen and the concentration of RNA during irradiation had an effect on the amount of breakage. When the concentration of nucleic acid was increased from 0.04 mg/ml to 0.2 mg/ml, the rate of breakage decreased. It was also found that removal of dissolved oxygen from the more dilute RNA solutions by bubbling with purified N_2 gas resulted in an equal decrease in the rate of breakage of the RNA. The least square line for these data indicates a D_0 for breakage of about $18 \cdot 10^5$ R (Fig. 3).

When increased RNA concentration and removal of oxygen were combined, the rate of breakage was further decreased only slightly, suggesting that maximum protection had been achieved. An experiment was also done in which these maximally protected RNA samples were kept at liquid nitrogen temperatures instead of dry ice temperatures during irradiation. This substantial decrease in the temperature of the samples during irradiation did not change the rate of breakage. The results of both experiments are plotted together in Fig. 4. The least squares line indicates a D_0 for breakage of $21 \cdot 10^5$ R.

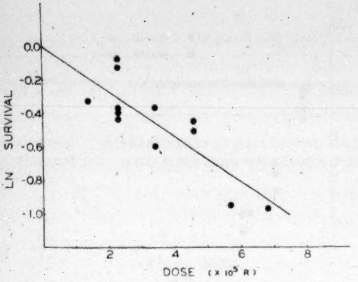

Fig. 2.

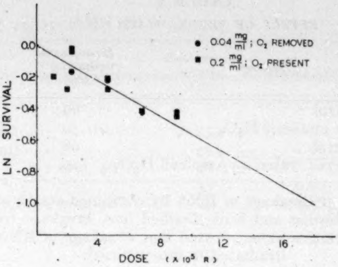

Fig. 3.

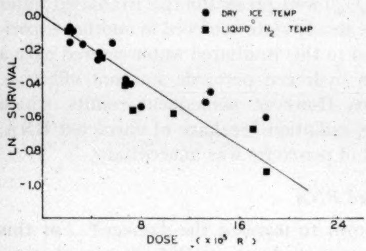

Fig. 4.

Fig. 2. Effect of X-irradiation on RNA in dilute solution.

Fig. 3. Effect of X-irradiation on RNA in concentrated solution or in oxygen free solution.

Fig. 4. Effect of X-irradiation on RNA in concentrated solution with oxygen removed.

Effect of hydrogen peroxide on RNA

The conditions that reduce the breakage of RNA, that is, removal of oxygen and increase of concentration, are standardly used to eliminate indirect action which results from the irradiation of water. It appeared anomalous that the radiation breakage of extracted RNA should in all cases be less rapid than breakage of RNA in the intact virus, which presumably is protected from indirect action[19]. A possible way that indirect action could affect the RNA in the whole virus is by the formation of organic peroxide groups on the protein[20]. These organic peroxide groups might then break the RNA later during the extraction procedure. For this reason the effect of hydrogen peroxide on RNA was studied. Storage of RNA with 2400 μmoles/l of hydrogen peroxide in the frozen state for several hours did not cause a drastic reduction of the amount of homogeneous RNA (Table I). A possible explanation is suggested by the reports that minute traces of stabilizer present in commercial peroxide reduce its effectiveness[21]. This has been questioned by CONWAY AND BUTLER[22], who showed that trace amounts of metallic-reducing catalysts are necessary for the activity of hydrogen peroxide on deoxyribonucleic acid. The virus from which the RNA was prepared was suspended in Versene, which would remove these catalysts from both commercial and irradiation-produced peroxides[23].

It is possible to measure the amount of peroxides produced in irradiated solution by using the oxidation of KI and determining liberated iodine (I^-_3) by optical absorption at 353 mμ (see ref. 24). The amount of peroxides produced when water was irradiated under various conditions was measured in this way. Distilled water was

TABLE I

EFFECT OF PEROXIDE ON RNA

Treatment	*Homogeneous material (%)*
Control	69
2400 μmoles/l H_2O_2	50
Control	68
X-rayed water, 45 μmoles/l H_2O_2	59

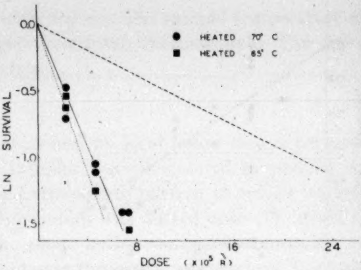

Fig. 5. Breakage of RNA by combined action of irradiation and heat. Dashed line, breakage by irradiation alone; dotted line, breakage of RNA irradiated in intact virus[1].

frozen with dry ice and irradiated for 12 h. The KI test showed, in agreement with data in the literature[25], that 45 μmoles of H_2O_2/l were present in this irradiated water after thawing, an amount about equal to the steady state achieved in another experiment in 1 h of irradiation at 0°. RNA added to this irradiated water showed only a small amount of breakage (Table I). Thus hydrogen peroxide was not effective. Organic peroxides might be more effective. However, subsequent results, which resolved the anomaly of the relatively slow radiation breakage of extracted RNA, indicated that further research on the effect of peroxides was unnecessary.

Heating and other post-treatments of irradiated RNA

Heating irradiated nucleic acids is known to increase the damage[26]. For this reason the following heating experiments were carried out on RNA after irradiation. After thawing, a portion of the irradiated sample was removed, diluted and centrifuged immediately; the remainder was used for heat treatment. For heating at 40° a 5-ml test tube containing 0.8 ml of buffer was placed in a water bath for several minutes to reach temperature equilibrium. The RNA was then added and the sample was heated with shaking for 1 or 2 min. The tubes contained approx. 4 times as much buffer as RNA. The heat treatment was stopped by plunging the tube into an ice bath. For the higher temperatures, test tubes containing 1-ml samples of diluted RNA solution were taken from an ice water bath and placed in the high temperature bath for 5 min. The heating was stopped by returning the samples to the ice water bath. The samples were centrifuged without further dilution. The results are shown in Table II. From Table II it can be seen that heating decreased the amount of material in the homogeneous component of the irradiated sample, but had little effect on unirradiated samples. Data for concentrated samples of RNA, irradiated with oxygen removed and heated at 65 or 70° after irradiation, are plotted in Fig. 5. The dashed line shows the breakage before heating. The solid line represents the least squares fit of the results from the heated samples and indicates a reduction in D_0 from 21 to $4.4 \cdot 10^5$ R. Thus, the breakage obtained after heating was very similar to that observed by ENGLANDER *et al.*[1] for the breakage of RNA extracted from irradiated TMV shown by the dotted line. Heating at 40° was done on RNA preparations irradiated under various conditions; therefore calculation of D_0 is not in all cases meaningful. Heating at 40° of a few samples of RNA irradiated at high concentrations with oxygen removed, yielded a value for D_0 of about $10 \cdot 10^5$ R.

It is possible that the observed breakage of RNA extracted from irradiated

TABLE II

EFFECT OF HEATING ON IRRADIATED RNA

Dose *($R \times 10^5$)*	*Conditions of irradiation*	*Heat treatment* *(time and temp)*	*Homogeneous material* *(%)*	*Surviving fraction*
Control	All samples at dry ice temperature and		45	
Control	with RNA concentration 0.2 mg/ml		45	1.00
Control		1 min, 40°	46	
1.1			37	0.82
1.1		1 min, 40°	26	0.58
2.0			34	0.75
2.0		1 min, 40°	19	0.42
Control			64	
Control		1 min, 60°	69	
Control		1 min, 60°	68	
Control			63	1.00
1.1			72	1.1
1.1		1 min, 40°	61	0.97
1.7			65	1.05
1.7		1 min, 40°	59	0.94
Control			56	
Control	Oxygen removed		55	1.00
2.3			52	0.93
2.3		2 min, 40°	41	0.73
5.7			48	0.86
5.7		2 min, 40°	36	0.64
13.7			36	0.64
13.7		2 min, 40°	17	0.30
Control	All samples with RNA concentration		61	
Control	0.2 mg/ml and O_2 removed		62	
Control		5 min, 65°	61	
Control			63	1.00
Control		5 min, 70°	63	
Control		5 min, 65°	63	
Control			66	
Control		5 min, 70°	64	
2.3	Liquid N_2 temperature		58	0.92
2.3		5 min, 70°	30	0.48
2.3		5 min, 65°	32	0.51
4.6			49	0.78
4.6		5 min, 70°	22	0.35
7.4			36	0.57
7.4		5 min ,70°	15	0.24
7.4		5 min, 65°	13	0.20
6.8	Dry ice temperature		42	0.67
6.8		5 min, 70°	15	0.24
Control			54	
Control			57	1.00
Control		5 min, 70°	50	
Control		5 min, 65°	48	
2.3	Dry ice temperature		51	0.98
2.3		5 min, 70°	32	0.61
2.3		5 min, 65°	31	0.60
4.6			46	0.89
4.6		5 min, 70°	17	0.33
4.6		5 min, 65°	15	0.29

virus might be the cumulative effect of radiation and the development of additional breakage by the extraction process. To test the effect of the extraction process, samples of irradiated RNA were treated in the same manner as the virus is treated to isolate the RNA. This process will be called "re-extraction". The results are shown in Table III. Re-extraction did not affect unirradiated RNA but increased the breakage of irradiated RNA. Actual contact with phenol must be necessary because, if solutions of irradiated RNA were shaken with ether and then bubbled with N_2 gas, the final steps in the extraction process, no increase in RNA breakage was observed. The breakage is not quite as great as breakage of the RNA extracted from the irradiated virus.

TABLE III

EFFECT OF POST-TREATMENTS ON IRRADIATED RNA

Dose *($R \times 10^5$)*	*Post-treatment*	*Homogeneous material* *(%)*	*Slower sedimenting material* *(%)*
Control		62	17
Control	Re-extraction	63	12
3.4		48	41
3.4	Re-extraction	37	55
6.8		42	35
6.8	Re-extraction	35	42
Control		56	22
Control	Shaken with ether	62	12
8.0		33	48
8.0	Shaken with ether	32	45

Irradiation of dried RNA

Irradiation of dried RNA was also studied. Shortly after extraction, 0.2 ml of the RNA preparation with concentration adjusted to 0.2 mg/ml in 0.02 *M* phosphate buffer was placed on a cover slip and dried in the frozen state. After drying, the samples were sealed between two sheets of parafilm by pressure. The dried RNA samples were cooled during irradiation to a temperature slightly below 25°. After

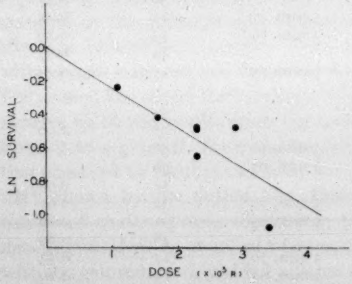

Fig. 6. Breakage of RNA irradiated in dry state.

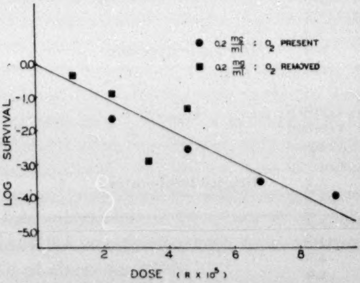

Fig. 7. Inactivation of infectious RNA.

irradiation, the samples were allowed to redissolve slowly in buffer at about 5° for 1 h: the final concentration was 0.04 mg/ml in 0.02 *M* phosphate buffer. Drying has negligible effect on the homogeneity of the RNA. The results of the X-irradiation of dried RNA, shown in Fig. 6, indicate a D_0 for breakage of $4.2 \cdot 10^5$ R. Therefore, radiation breaks dried RNA apparently as fast as the RNA in the virus. Radiation breakage of dried RNA presumably would be almost completely caused by direct effect.

Biological inactivation of RNA

The biological activity of infectious RNA from TMV is also destroyed by X-rays[18]. RNA was irradiated at a concentration of 0.2 mg/ml with and without treatment to remove oxygen. The results are plotted together in Fig. 6 because they are only slightly different for these two conditions. The D_0 for inactivation was found to be about $1 \cdot 10^5$ R. It is substantially lower than the D_0 for the breakage of RNA under any of the conditions used; however, it is nearer the D_0, $1.8 \cdot 10^5$ R, for inactivation of the parent virus[1]. GINOZA AND NORMAN[18] found that D_0 for inactivation of RNA was $3 \cdot 10^5$ R for long wave length X-rays[19].

GIERER AND SCHRAMM[26] investigated the infectivity of nucleic acid solutions prepared by the phenol method and concluded that the infectivity could not be the result of TMV contamination. It has been shown that the infectivity of various RNA batches prepared in this laboratory by the phenol method was not caused by residual virus[28], but such tests were not performed on the individual RNA preparations used in these inactivation studies.

DISCUSSION

Treatment with heat or phenol after irradiation develops breakage not previously observed. Such mild after-treatment has little effect on unirradiated RNA. DOTY *et al.*[2] have proposed that as many as 60 % of the bases of the single stranded RNA molecule are hydrogen bonded to form a system of closed loops. It is possible that these bonds are strong enough to hold two segments of a loop together even though radiation had produced a break somewhere in the main chain. DOTY found that the hydrogen bonding could be eliminated by heating. Thus the development of breaks by heating irradiated RNA might result from allowing the broken parts to separate by elimination of intramolecular hydrogen bonds. Phenol also could break the hydrogen bonds.

The data of DOTY *et al.*[2] indicate that some hydrogen bonds are broken even at 40° and that practically all are eliminated at 65°. From the results of our experiments it can be estimated that heating irradiated RNA at 40° reduced the D_0 for breakage from $21 \cdot 10^5$ R to about $10 \cdot 10^5$ R and that heating at 65 or 70° reduced the D_0 to $4.4 \cdot 10^5$ R, a value in good agreement with the D_0 of $4.0 \cdot 10^5$ found[1] for the breakage of RNA irradiated in the intact virus. These data are therefore in good agreement with the hydrogen bonded loop hypothesis. The rate of breakage of dried RNA is also similar to that observed for irradiated RNA heated at 65 or 70°. In this case an unfolding of the RNA molecule might occur because of the sharp reduction in dielectric constant when the RNA goes from a water medium to an air medium.

Breakage of RNA extracted from irradiated virus is closely similar in extent to breakage of irradiated free RNA after heating. In addition it has been found that breakage cannot be comparably increased by heating the RNA extracted from irradiated virus[29]. This can be explained by the assumption that when RNA is held in a rigid configuration in the intact virus it is unable to hydrogen bond to itself in the form of closed loops and therefore all breaks are observed on extraction. It is concluded that RNA breakage in the whole virus is not an artifact of the extraction procedure, but an accurate indication of the direct radiation action.

The D_0 found for the inactivation of infectious RNA was $1 \cdot 10^5$ R which is slightly lower than the D_0 of $1.8 \cdot 10^5$ R found by Englander *et al.*[1] for the inactivation of TMV. It is not unreasonable to expect that some products of indirect action might have an effect on the isolated RNA. While there was no observed difference between the inactivation rate in the presence or absence of oxygen, production by indirect action of free radicals not affected by oxygen concentration could result in some extra damage.

ACKNOWLEDGEMENT

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THE EFFECT OF MUTATION ON RIBONUCLEIC ACID, PROTEIN AND RIBONUCLEASE FORMATION IN *NEUROSPORA CRASSA*

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SUMMARY

Electrophoretic and biochemical analyses have been carried out to determine whether gene mutation in *Neurospora crassa* can elicit gross changes in nucleic acid or protein formation. Alterations in ribonucleic acid, ribonuclease and total protein formation which were observed during growth could not be specifically attributed to gene mutation.

The ribonuclease of *N. crassa* resembles ribonucleases from other sources in many of its properties. However, the *Neurospora* enzyme can be completely inhibited by ethylenediaminetetraacetic acid.

INTRODUCTION

That specific gene mutations can cause changes in the properties of specific enzymes is well known. During the past decade, a number of such instances have been noted, and certain of these have been studied in detail[1-3]. All of the cases studied to date, however, relate to minor components of the total protein formed by the organism. As enzyme formation is genetically determined, the question arises—does a gene change resulting in an alteration of a specific enzyme affect only this enzyme, or are there additional macromolecular changes associated with the genetic damage which are not detected by the usual screening methods, *i.e.*, can genetic change give rise to a gross alteration in the protein or nucleic acid composition of a cell? An answer to this problem has been sought by establishing the electrophoretic pattern of extracts of wild type *Neurospora crassa* and comparing this with the pattern found in certain mutants. No changes in protein maxima were observed, but a difference in a ribonucleic acid component was noted. Further experiments indicated that abnormal electrophoretic patterns can result from differences in growth rate but that no major differences in protein and nucleic acid components occur which are specifically attributable to single gene mutation. In the present paper this work will be summarized. In the course of these investigations, however, a good deal of detailed

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Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; ATP, adenosine triphosphate; EDTA ethylene diaminetetraaceticacid; TCA, trichloroacetic acid.

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data concerning the electrophoretic characterization of *Neurospora crassa* extracts and the properties of *Neurospora* ribonuclease have been compiled. Not all of the data will be recorded here, and the interested reader is referred to the paper by SUSKIND[4].

MATERIALS AND METHODS

Strains

The cultures were grown either as stationary cultures in 2-l Fernbach flasks containing 500 ml of minimal medium plus 2 % sucrose[5], or as aerated cultures grown in 5-gallon carboys. All wild type and morphological mutant cultures were grown on a minimal medium plus 1 % glucose or 2 % sucrose. Biochemical mutant strains were grown on minimal medium supplemented with the appropriate nutrient. After growth, the mycelia were harvested, rinsed with distilled water and stored at —20°, or the mycelia were immediately lyophilized and the lyophilized mycelia were stored at —20°.

Preparation of extracts for analysis

Extracts suitable for electrophoretic analysis were prepared as follows: 500 mg of lyophilized mycelia were ground to a fine powder with a mortar and pestle. The powder was extracted with 10 ml of cold 0.1 *M* phosphate buffer, at pH 7.5–7.8 by shaking with glass beads in the cold for 1–2 h. The extracts were centrifuged at about 3200 rev./min for 0.5 h. The supernatant solutions were subsequently centrifuged for 60 min in a Spinco Model L Preparative Ultracentrifuge at 55,150 × *g*. The clear supernatant solutions were withdrawn using a needle and syringe.

Nucleic acid and protein determinations

All extracts were either stored at —20° or analyzed immediately for nucleic acid and protein content. Ribonucleic acid was estimated by the SCHNEIDER technic[6] using orcinol and u.v. absorption methods for determining ribose and base respectively. DNA was measured by the DISCHE cysteine–HCl method[7] following the procedure described by STUMPF[8]. Protein was determined by the biuret, micro-Kjeldahl or LOWRY methods[9–11].

Electrophoretic analysis

Dialysis was carried out in an Aminco internal stirring mechanical dialyzer at 2°. Three electrophoretic buffers, covering the pH range 6.9–10.0 were used with satisfactory results: cacodylate buffer, pH 6.9, $\mu = 0.1$; veronal buffer, pH 8.6, $\mu = 0.1$; and ammonia buffer, pH 10.0, $\mu = 0.1$ (see ref. 12–14). An Aminco-Stern electrophoresis unit was employed and the final protein concentration of the extracts examined was ordinarily between 1 % and 1.5 %.

Determination of ribonuclease activity

Ribonuclease activity was assayed using yeast RNA (Schwarz Laboratories) as substrate, in the presence of 0.1 *M* phosphate buffer, pH 7.5 at 37°. A 2 % solution of yeast nucleic acid in distilled water (containing 0.84 % acid precipitable nucleic acid), adjusted to pH 6.0, served as the stock from which suitable aliquots were taken. Activity was measured by following the decrease in acid-precipitable nucleic acid using the SCHNEIDER method[6]. The samples were analyzed for pentose by the orcinol method and/or for base by their absorption at 260 mμ.

A unit of ribonuclease activity is defined as the amount of enzyme which depolymerizes 1.0 mg of RNA in 30 min at 37°.

The dry weights, ribonucleic acid and protein content of the lyophilized mycelia are expressed in mg per pad, while ribonuclease activity is given in units of enzyme activity per pad.

EXPERIMENTAL

Electrophoretic results: wild-type studies

Experiments were carried out employing several wild-type strains to establish the normal electrophoretic pattern of *Neurospora crassa* extracts and to determine the effect of culture and extraction conditions on this pattern.

A constant and characteristic electrophoretic pattern was found for extracts of various wild-type strains grown, prepared and tested under a variety of conditions.

Of the four maxima observed, only the fastest moving acid component was isolated and identified. This component had a mobility approximating that of ribonucleic acid. Employing a sampling apparatus, small amounts of this component were withdrawn from the ascending limb of the electrophoretic cell. Ribonucleic acid, DNA and protein determinations were run on the sample and the nucleic acid/protein ratio of the fraction and of the extract were compared. These data together with relative concentration measurements of the maxima are presented in Table I. Component 1, comprising approx. 4% of the total area, consists predominantly of RNA, the RNA/protein ratio of component 1 being 26 times higher than the crude extract value. No DNA was detected in this fraction.

TABLE I

ELECTROPHORETIC MAXIMUM NO. 1 IN WILD TYPE *N. crassa* EXTRACTS

Mobilities expressed as $\mu \times 10^{-5}$ cm²/sec^{-1}, V^{-1}.

Electrophoretic maximum No.	*Limb*	*Mobility*	*Relative concentration %*	*mg RNA / ml*	*mg protein / ml*	*mg RNA / mg protein*
1	Asc.	−11.2	3.9	0.134	0.038	3.54
	Desc.	− 9.0	4.2	—	—	—
2	Asc.	− 7.1	—	—	—	—
	Desc.	− 5.4	Combined 2, 3	—	—	—
			(asc.) 78.0			
3	Asc.	− 6.1		—	—	—
	Desc.	− 2.7	(desc.) 76.8	—	—	—
4	Asc.	0	12.1	—	—	—
	Desc.	0	12.8	—	—	—
Total extract (undialyzed)		—	—	2.1	15.5	0.136

Mutant studies

Several morphological mutants were selected for comparison with the wild-type.

Those mutants were chosen which showed the most striking aberrancies in growth and physical appearance, since it seemed more likely that gross electrophoretic changes might be discernable in this group. Electrophoretic patterns were obtained which indicated a major change in the number of maxima present in extracts of two colonial mutants, Y-9833a and Y-7381 and a microconidial strain, JH-146 (see ref. 15).

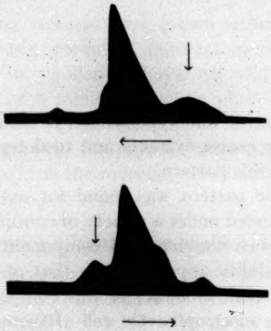

Fig. 1. The electrophoretic pattern of an extract of wild type *N. crassa*, strain 5265A; 5040 sec migration in 0.1 μ veronal buffer, pH 8.6 at a potential gradient of 5.0 V/cm; 1.5 % total protein.

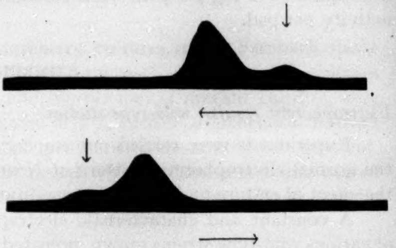

Fig. 2. The electrophoretic pattern of an extract of *N. crassa* colonial mutant, strain Y-7381; 2580 sec migration in 0.1 μ veronal buffer, pH 8.6, at a potential gradient of 8.0 V/cm; 1.1 % total protein.

Extracts of the mutants characteristically lacked the ribonucleic acid-containing component present in the wild-type preparations. This is shown in Fig. 2. A tryptophan-requiring mutant (td_2) and a tryptophan-independent suppressed mutant (td_2su_2) were tested and extracts of these strains exhibited the same pattern noted in the morphological mutants.

The ribonuclease of Neurospora crassa

A substantial decrease in the RNA content of *Neurospora* extracts occurred during extraction and dialysis prior to electrophoresis. This loss was due in part to the action of ribonuclease. Experiments were carried out to characterize the ribonuclease of *N. crassa*, to establish the conditions necessary for inhibition of this activity in the extracts and to determine whether the loss of the RNA component in the mutant extracts represented an RNA difference associated with genetic change, with growth or with an altered or enhanced ribonuclease activity.

Extracts were prepared and assayed for ribonuclease activity as described in the section on MATERIALS AND METHODS. Ultracentrifugation of crude extracts at 41,190 × *g* for 90 min, did not sediment ribonuclease activity. No experiments were carried out on the 100,000 × *g* fraction which would presumably have contained the ribosomes[16].

An inhibitory effect of ribonucleic acid on *Neurospora* ribonuclease was noted which was similar to the results reported by LAMANNA AND MALLETTE[17] for crystalline pancreatic ribonuclease. The effect of substrate concentration. on activity is shown in Table II. The enzyme is inhibited by the products of RNA depolymerization; however, under the conditions of the assay, activity is linear with time for 30 min.

Attempts to inhibit the enzyme with purine and pyrimidine bases, or with nucleosides and nucleotides, were unsuccessful at concentrations as high as $10^{-2} M$, with the exception of ATP, which was inhibitory at $8 \cdot 10^{-3} M$.

Maximum activity is found at pH 7.5 with about 75% loss in activity at pH 6.0. Total inactivation occurs at pH 9.2. An increase in phosphate from 0.2 *M*–0.5 *M*, results in a loss of activity, a 45% reduction occurring at 0.5 *M*. Maximum activity is found at 60° in 0.1 *M* phosphate buffer. The enzyme is resistant to elevated temperatures for prolonged periods of time which should prove of considerable value in purification.

TABLE II

THE EFFECT OF RNA CONCENTRATION ON THE ACTIVITY OF *N. crassa* RIBONUCLEASE

Tubes contained 0.2 ml *N. crassa* extract; RNA (20 mg/ml stock solution); 0.1 *M* PO_4 pH 7.5. Total volume per tube equaled 1.2 ml. Incubation was for 30 min at 37°.

mg RNA/tube	*% RNA hydrolyzed*	*mg RNA hydrolyzed*
0.8	76	0.608
1.6	60	0.960
2.4	50	1.20
3.2	42	1.34
4.0	26	1.04
6.0	2	0.01

Dialysis of the enzyme preparation causes no loss in activity, eliminating the likelihood of a readily dialyzable prosthetic group. Almost all of the divalent ions employed at concentrations of $10^{-2} M$ caused inhibition of the enzyme. The most effective inhibitors at $10^{-3} M$ were copper and cobalt. Both ferrous and ferric ions were inhibitory.

The purine analogues benzimadazole and 2-amino benzimadazole[18] were also tested as inhibitors. Benzimadazole at a concentration of $10^{-2} M$ caused no reduction in activity, while the 2-amino derivative was moderately inhibitory. The possibility of inhibiting *Neurospora* ribonuclease activity with EDTA at several pH values was examined. The inhibitory effect of EDTA is most effective at pH 7.0. The chelating action of EDTA is known to be most effective at pH 7.0, which corresponds to the pH dependence of EDTA inhibition.

The fact that EDTA inhibits the action of *Neurospora* ribonuclease was used to prevent the loss of RNA during extraction,dialysis and electrophoretic analysis. As seen in Table III, the presence of $6 \cdot 10^{-3} M$ EDTA inhibits the breakdown of Neurospora RNA 57%. At 0.1 *M* EDTA, no significant ribonuclease activity remains. The inhibitory effect of EDTA can be almost completely reversed by dialysis against 0.05 *M* phosphate buffer, pH 7.8, for 24 h.

The addition of EDTA during dialysis permitted the detection of a small RNA electrophoretic component in the mutant extract. In the absence of EDTA, under identical conditions, there was a 42% drop in the RNA/protein ratio and no component was visible. It should be emphasized that a comparison of the RNA/protein ratios of undialyzed mutant and wild-type extracts, freshly prepared from cultures of approximately equal age, gave a mutant ratio which was 40–70% below the

TABLE III

THE EXTRACTION OF *N. crassa* RNA AND PROTEIN
THE EFFECT OF EDTA DURING EXTRACTION ON THE RATE OF DEPOLYMERIZATION OF *Neurospora* RNA

Preparation	*EDTA Ratio*	*Minutes incubated at 37°*					
		10		*20*		*30*	
		mg RNA hydrol.	*% inhibit*	*mg RNA hydrol.*	*% inhibit*	*mg RNA hydrol.*	*% inhibit.*
Control — no EDTA	—	0.790	—	1.20	—	1.48	—
EDTA $6 \cdot 10^{-3}$ *M*	1	0.333	58	0.486	60	0.640	57
EDTA $3 \cdot 10^{-2}$ *M*	5	0.181	77	0.286	76	0.364	75
EDTA $6 \cdot 10^{-2}$ *M*	10	0.082	90	0.166	86	0.166	89
EDTA 0.12 *M*	20	0.02	97	0.02	97	0.02	97

TABLE IV

RNA, PROTEIN AND RIBONUCLEASE FORMATION IN WILD TYPE STRAINS OF *N. crassa*

Strain	*Age (h)*	*mg dry wt./pad*	*mg RNA / pad**	*mg protein / pad**	*mg RNA / mg protein*	*Ribonuclease units/pad**
5256A	36	170	13.1	60.0	0.219	18.3
	48	355	18.1	106.0	0.171	32.6
	96	1030	39.2	252.0	0.155	62.0
	192	1800	64.7	396.0	0.163	172.0
Chilton a	48	410	18.8	93.5	0.201	31.2
	72	900	32.3	173.0	0.187	46.9
	96	1300	40.3	228.0	0.177	41.5
	192	1800	64.7	346.0	0.187	93.5

* 1. $\text{mg RNA or protein per pad} = \dfrac{\text{mg RNA or protein/ml of extract}}{\text{mg lyophilized mycelia/ml of extract}} \times \text{dry wt. of pad.}$

2. $\text{ribonuclease units per pad} = \text{ribonuclease units/ml of extract} \times \dfrac{\text{mg lyophilized mycelia per pad}}{\text{50 mg lyophilized mycelia/ml of extract}}$

TABLE V

RNA, PROTEIN AND RIBONUCLEASE FORMATION IN MUTANT STRAINS OF *N. crassa*

Strain	*Age (h)*	*mg dry wt./pad*	*mg RNA / pad**	*mg protein / pad**	*mg RNA / mg protein*	*Ribonuclease units/pad**
Y-9833a	48	340	12.9	76.5	0.169	13.6
	96	1170	31.6	206.0	0.153	18.8
	192	1260	27.7	212.0	0.131	10.1
C-83a	48	150	7.6	42.0	0.181	9.5
	72	430	17.2	110.0	0.156	27.0
	120	890	39.8	266.0	0.149	63.5
	168	970	43.3	300.0	0.143	74.0
	192	1100	41.0	332.0	0.123	61.0
10575	48	165	5.8	24.4	0.238	8.0
	96	1920	32.6	107.0	0.157	145.0
	192	2120	25.4	186.0	0.137	76.5

* See under Table IV.

wild-type value. Hence, at least two factors play a role in resolving the RNA electrophoretic component in the mutant extracts: the initial RNA/protein ratio of the sample and the extent of RNA depolymerization by ribonuclease during the preparative procedure.

Growth studies

The ribonucleic acid, protein, and ribonuclease concentrations of several wild-type and mutant strains were examined at intervals during an 8-day growth period. A comparison of the data from wild-type strains 5256A and Chilton A is shown in Table IV. The RNA and protein values for the two strains appear to be in good agreement. It can be seen that neither culture has reached maximum growth at 192 h, and that RNA and protein formation appear to be roughly proportional to the rate of growth. It can also be seen from the 36 and 48-h values, that the maximum RNA/protein ratios occur in young cultures. Between 72 and 192 h, this ratio changes very little. The ribonuclease activities of the two strains, although quantitatively different, nevertheless increase with age.

Colonial mutant Y-9833a, and tryptophan mutants td_1[19] and 10575 behaved in an entirely different manner. These data are presented in Table V. It can be seen that the net synthesis of RNA and protein reached a maximum in two of the mutants, Y-9833a and 10575 at about 96 h. In td_1 the RNA value leveled off at about 120 h while protein formation continued. In all of these mutants, the RNA/protein ratio began to decrease significantly once the period of maximum RNA synthesis had been attained. Again, the highest RNA/protein ratios were observed in the young cultures.

The course of ribonuclease formation also differed from the wild-type strains. In the mutants, either ribonuclease activity reached a maximum or sharply decreased concurrent with the decrease in RNA synthesis. The decrease in intracellular ribonuclease activity, which occurred in some cases when the cultures had attained maximum growth, suggested that the enzyme might be released into the culture medium during this period. Aliquots of filtrates of several strains were tested for ribonuclease activity and the extracellular and intracellular enzyme values were compared. As seen in Table VI, the culture filtrates contain considerable ribonuclease activity. This activity in general appears to be maintained at a low level for about 3–5 days and then begins to increase as the culture ages. This increase in extracellular ribonuclease activity has been observed even after maximum RNA formation has been attained. The appearance of extracellular ribonuclease activity is not accompanied by a significant change in the 280 mμ/260 mμ ratio in the filtrate nor by the appearance of TCA precipitable material. Hence, it would appear unlikely that large-scale autolysis had occurred during the course of these growth experiments.

Since these data suggest that certain mutations may affect jointly the course of RNA, protein and ribonuclease formation, it was of considerable importance to determine whether a mutant possessing the ability to grow at wild-type rate would have the wild-type pattern of RNA, protein and ribonuclease formation during an 8-day growth period. An inositolless mutant, WS-7336, exhibited essentially wild-type growth rate when grown on minimal medium plus 10 μg/ml inositol and in this instance ribonucleic acid, protein and ribonuclease formation occurred in a typically wild-type manner. Furthermore, the changes observed in most of the aberrantly growing mutants could be elicited in a non-mutant strain. It is known that colonial-type

TABLE VI

INTRACELLULAR AND EXTRACELLULAR RIBONUCLEASE ACTIVITY IN *N. crassa*

Strain	*Age (h)*	*Ribonuclease units/pad*	*Total filtrate ribonuclease units**	*Extracellular/intracellular activities*
C-83 (td_1)	72	27.0	164.0	6.0
	120	63.5	234.0	3.7
	168	74.0	312.0	4.2
	192	61.0	432.0	7.1
S-1952 (td_2)	72	25.6	247.0	9.1
	120	42.5	650.0	15.5
	168	80.0	1170.0	14.7
	192	96.5	1380.0	14.4
Y-9833a	72	24.3	208.0	8.5
	120	36.5	354.0	9.7
	192	47.0	505.0	11.0

* Total filtrate ribonuclease units = ribonuclease units/ml of filtrate × total volume of filtrate after pads have been harvested.

TABLE VII

THE EFFECT OF SORBOSE ON RNA, PROTEIN AND RIBONUCLEASE FORMATION IN WILD TYPE STRAIN 5256A

Strain	*Age (h)*	*mg dry wt./pad*	*mg RNA / pad*	*mg protein / pad*	*mg RNA / mg protein*	*Ribonuclease units/pad*
5256A	48	105	6.7	34.0	0.197	8.4
	72	450	22.2	139.0	0.159	26.0
	120	570	15.5	125.0	0.124	68.0
	168	750	13.0	127.0	0.102	105.0
	192	770	7.6	92.0	0.083	132.0

growth can be induced in wild-type strains of *N. crassa* by employing suitable ratios of sorbose and sucrose in the medium[20]. Using 1 % sorbose, and 0.5 % sucrose, an altered growth response of strain 5256A was observed. As shown in Table VII, a marked difference was also observed with respect to RNA, protein and ribonuclease formation.

DISCUSSION

The results of growth experiments with *Neurospora* and with other organisms[21–23] show that a high RNA/protein ratio is obtained in actively growing cells, while in old cells the ratio decreases significantly. Consequently a likely explanation for the altered electrophoretic pattern and the low RNA/protein ratio in certain *Neurospora* mutants rests in their modified growth rate. In certain mutant strains, RNA, protein and ribonuclease net synthesis terminate prematurely. This is accompanied by a decrease in the RNA/protein ratio of the extract, and by an increase in extracellular ribonuclease activity.

The low RNA/protein ratios found in older cultures might be accounted for in two ways: either by a diminished rate of RNA synthesis, or by an actual loss of

RNA in the mycelia. As the growth rate decreases there is a concomitant decline in intracellular ribonuclease activity, while the extracellular activity increases. It does not appear that autolysis of the culture can account for these results. It is conceivable that the increase in extracellular ribonuclease activity and the decrease in intracellular RNA may reflect a liberation or activation of the intracellular enzyme at certain sites in the cell, *i.e.*, the ribosomes[16].

The ribonuclease from *Neurospora crassa* appears similar in several respects to ribonucleases from other sources[24]. Activity is inhibited both by high substrate concentration and by the accumulation of the products of RNA depolymerization. No loss of activity occurs as a result of dialysis, and the enzyme is heat-resistant. It appears that the inhibition of *Neurospora crassa* ribonuclease by the chelating agent EDTA is unique. This inhibition is pH dependent and can be reversed by dialyzing the inhibited enzyme. No metal requirement for the enzyme can be demonstrated.

The fact that *Neurospora* ribonuclease activity is heat-resistant, and that it can be obtained from both cells and culture filtrates, would make possible its rapid purification. An extensive purification of this enzyme from *Neurospora* has been recently undertaken by SINGER *et al.*[25] and the sedimentation constant suggests a molecular weight similar to pancreatic ribonuclease.

No evidence was obtained for a qualitative difference between ribonuclease from wild-type and mutant strains, but such a difference might be found by suitable screening procedures. In view of the available information on ribonuclease structure and function[24–27], mutants of this type could prove most informative in studies on gene–enzyme relationships at the molecular level.

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THE NUCLEIC ACID CONTENT OF *ESCHERICHIA COLI* STRAINS B AND B/R

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SUMMARY

The differences in the DNA and RNA content of cells of *Escherichia coli* strain B and its radio-resistant mutant, strain B/r, in the logarithmic phase of growth are not statistically significant. In the stationary phase, cells of strain B/r contain more DNA than those of strain B, but the difference is barely significant; the RNA content of the B/r cells is significantly higher. The differing radiosensitivities of the two strains cannot be correlated with differences in their content of nucleic acids.

INTRODUCTION

In recent years considerable emphasis has been placed on the possible connection between DNA and the specific site of damage in living cells exposed to ionizing or u.v. radiations. It is sometimes assumed that the lethal effect of radiation (*i.e.* inhibition of clone formation) is primarily due to damage to the DNA itself. Direct correlation between the DNA content of cells and their radiosensitivity might support this assumption, although lack of correlation is, of course, not evidence against it. Several authors have attempted to relate radiosensitivity to DNA content. Thus Pardee and Prestidge[1] concluded that a relative surplus of DNA over protein and RNA in cells of *E. coli* B was probably responsible for reducing the u.v. sensitivity of bacteria which had been treated with β-2-thienylalanine. Billen[2] implied that the reduction of X-ray sensitivity, conferred by incubating *E. coli* B/r and *E. coli* $15T^-$ with chloramphenicol before irradiation, was due to an increase in the DNA/protein ratio of the cells. These papers exemplify the line of reasoning that the radio-

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Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

sensitivity of cells of the same strain can be negatively correlated with their DNA content, and that if a radio-resistant mutant develops from a parent strain, this can be attributed to the greater DNA content of the mutant. On the other hand, it is possible to argue, as PUCK[3] has done, that the more DNA there is present in a cell the greater will be the target presented to the damaging radiation, and therefore radiosensitivity will increase with an increase of DNA content.

A well known example of a radio-resistant mutant is the strain B/r isolated by WITKIN[4] from *E. coli* strain B, and the report by MORSE AND CARTER[5], that *E. coli* B/r cells contain more DNA than those of *E. coli* B, has frequently been quoted. The relevance of the nucleic acid content of *E. coli* B and its mutant to their differing radiosensitivities became very questionable when HAROLD AND ZIPORIN[6] were unable to confirm the results of MORSE AND CARTER[5], and reported in contrast that the concentration of DNA in the two strains was the same. These conflicting results have led us to examine the nucleic acid contents of the two strains, in different physiological stages, paying particular attention to the errors which arise in the measurements.

METHODS

Bacteria

Escherichia coli strain B and *Escherichia coli* strain B/r were maintained on slopes of Oxoid Blood Agar Base.

Growth of cells

A loopful from the stock slope was inoculated into 5 ml of Oxoid nutrient broth, which was then incubated, without aeration, for 18 h at 37°. This culture, containing approximately $5 \cdot 10^8$ cells/ml, was used to prepare batches of about 10^{10} logarithmic or stationary-phase cells.

Logarithmic-phase cells: A sample of the stock broth culture, containing about $5 \cdot 10^7$ cells, was added to each of 8 × 100 ml screw-cap bottles half filled with Oxoid nutrient broth. The bottles were incubated for 2.5 h under the aeration provided by incubating at 37° on a turntable rotating at an angle (Matburn Ltd., London). The cultures were cooled in melting ice, and the cells were centrifuged at room temperature, and resuspended in 20 ml of 0.067 *M*, pH-7 phosphate buffer.

Stationary-phase cells: Two bottles, inoculated from the stock broth culture, were incubated, with aeration, for 24 h at 37°, and the cells harvested as above.

Estimations of errors in analysis

In the chemical estimation of the concentration of any cellular constituent, errors are involved in both the counting of the cells and the chemical estimation.

Counting of cells: Both total and viable cell counts were made. For total counting, three unequal dilutions of the concentrated cell suspension were made in phosphate buffer, containing 1% formalin to prevent further cell division. These were coded so that the relative cell concentrations were unknown to the observer, who used a calibrated Helber bacterial counting chamber (Hawksley & Sons Ltd., London) to count the cells. The weighted mean of the three counts was used in estimating the nucleic acid content per cell, and the average errors in the counts were estimated from the error in each set of three counts.

DNA and RNA estimations: The concentrated suspension of cells was divided into three unequal parts, which were subsequently treated alike. The means and the standard errors of the nucleic acid estimations were calculated from the three values obtained for each batch. The cells were removed from the buffer by centrifugation and the nucleic acids extracted with perchloric acid according to the method of BURTON[7]. The concentration of DNA in the acid extract was determined by its reaction with indole and HCl, by the method of DISCHE[8] as modified by CERIOTTI[9]. RNA was estimated with orcinol according to the modification of the method of BARRENSCHEEN AND PEHAM[10] introduced by CERIOTTI[11]. Reference standards were prepared from samples of DNA and RNA purchased from the Nutritional Biochemicals Corporation.

RESULTS

The standard error in chemical estimation of both DNA and RNA was within 4 % of each mean estimate, assuming that a normal error curve applied to the three values obtained for each batch.

From 51 individual counts made in the series of experiments, it was determined that the standard error in making a count of *n* cells was $\sqrt{2n}$. Since a total of about 1200 cells was counted from each batch used in the chemical determinations, the average value of 4 % was adopted as the standard error of counting. The importance of basing estimates of any cellular constituent on total and not on viable count is demonstrated in Table I.

TABLE I

THE NUCLEIC ACID CONTENT PER CELL OF *E. coli* STRAIN B AND *E. coli* STRAIN B/r IN TWO DIFFERENT STAGES OF THE GROWTH CYCLE

Strain	*Time of incubation (hr)*	*% viable cells*	*DNA ($\times 10^{-8}$ μg/cell)*	*RNA ($\times 10^{-8}$ μg/cell)*	*RNA/DNA*
E. coli B	2.5	93	1.53	8.66	5.68
		48	1.12	6.24	5.59
		72	1.46	8.63	5.91
Mean for group			1.37 ± 0.13	7.84 ± 0.79	5.73 ± 0.10
E. coli B/r	2.5	60	1.31	7.00	5.46
		69	1.78	12.60	7.12
		74	3.03	13.80	4.58
		—	1.38	8.79	6.39
		71	1.60	8.22	5.18
Mean for group			1.82 ± 0.30	10.08 ± 1.32	5.75 ± 0.45
E. coli B	24	16	0.70	0.65	0.92
		11	0.71	0.68	0.93
		12	0.92	0.62	0.68
Mean for group			0.78 ± 0.07	0.65 ± 0.02	0.84 ± 0.09
E. coli B/r	24	42	1.16	1.96	1.69
		45	1.13	2.53	2.23
		18	1.54	1.54	1.00
Mean for group			1.28 ± 0.13	2.01 ± 0.30	1.64 ± 0.36

The concentrations of DNA and RNA found per cell of *E. coli* B and *E. coli* B/r in different stages of growth are shown in Table I. The value given for the content of DNA and RNA per cell in each batch is the mean of three determinations. The errors involved in counting the cells and in the chemical estimations are both included in the standard error attached to the mean value, which standard error is based on the variance of the three or more values whose mean has been taken. The results within each group show that the dispersion of the values between batches was greater than could be accounted for by combining the estimated errors in chemical procedures and in counting the cells. Although all batches of cells within each group were grown and harvested as far as possible in the same way, it is clear that uncontrolled variations arose from batch to batch. The largest number of batches examined in any one group was five (for logarithmic-phase cells of B/r), which is not large enough to determine the type of distribution of the values obtained. However, the values within this group are consistent with the assumption that there is a normal distribution of errors, and therefore the standard error of the mean value for each group has been calculated on this basis.

Comparisons were made between the group means for the DNA and RNA contents of the two strains both in the logarithmic and the stationary stages of growth, and also between the DNA and RNA contents of cells of the same strain in the two physiological stages. An analysis of variance was used to test the significance of differences between pairs of means. These comparisons show that (a) the differences in the DNA and the RNA content of the two strains in the logarithmic phase were not significant at $P = 0.05$ level; (b) the concentration of DNA in stationary-phase cells of *E. coli* B/r was just significantly higher than that in *E. coli* B. ($P = 0.05$); (c) there was significantly more RNA in stationary-phase cells of strain B/r than in strain B (significant at $P = 0.05$ level and almost significant at $P = 0.01$); (d) the difference between the DNA contained in logarithmic-phase and stationary-phase cells of strain B was just significant ($P = 0.05$); (e) there was no significant difference in the DNA content of B/r cells in the different physiological states.

The ratios of RNA/DNA, which are rather more accurate as they are independent of counting errors, were the same for logarithmic-phase cells of the two strains, but this ratio was significantly higher for resting cells of strain B/r than for those of strain B. For both strains the absolute quantity of RNA, and the RNA/DNA ratio were very much greater in logarithmic than in stationary-phase cells. This increase in the RNA concentration in actively dividing bacterial cells has been reported by many authors (*e.g.* 12–14).

DISCUSSION

Few values for the nucleic acid contents of *E. coli* B and *E. coli* B/r are available in the literature. The only study comparable to our own was made by HAROLD AND ZIPORIN[6] on cells grown in a synthetic medium, and their results are shown in Table II. Their figures for DNA and RNA are very similar to our results for logarithmic-phase cells, but their values for these in stationary-phase cells are much higher throughout. The differences in the latter are probably due to the fact that HAROLD AND ZIPORIN[6] based the concentrations per cell on the viable and not on the total cell count of their cultures. Our results show that the viable cell count is frequently much lower than the total cell count, especially in populations of non-dividing cells. However, in general, the results of HAROLD AND ZIPORIN[6] are confirmed by our findings, that there is no significant difference in the concentration of DNA in cells of the two strains in the same stages of growth. Figures kindly made available to us by HILL[15] again show (Table II) that the DNA and RNA contents of stationary-phase cells of strain B and strain B/r grown in nutrient broth are not appreciably different, although she found a higher content of RNA in *E. coli* B and a lower content of DNA in strain B/r than we have estimated.

MORSE AND CARTER[5] stated that cells of strain B/r contained 3–4 times the concentration of DNA in strain B, but they did not provide data in support of this, and the values which they quoted, for resting-stage cells grown in nutrient broth, do not show such a large difference (Table II). It is surprising that they found so much more DNA than RNA in stationary-phase cells of both strains.

TABLE II

COMPARISON OF THE RESULTS OBTAINED BY DIFFERENT WORKERS FOR THE NUCLEIC ACID CONTENT OF *E. coli* STRAIN B AND *E. coli* STRAIN B/r

Values are expressed as 10^{-8} μg of DNA or of RNA per cell.

			Present work	HAROLD AND ZIPORIN *(1958)*	HILL *(1960)*	MORSE AND CARTER *(1949)*
Logarithmic-phase cells	*E. coli* B	DNA	1.37	1.6–1.8	—	—
		RNA	7.84	10.0–12.0	—	—
	E. coli B/r	DNA	1.82	1.6–1.8	—	—
		RNA	10.08	10.0–12.0	—	—
Stationary-phase cells	*E. coli* B	DNA	0.78	2.4–2.6	0.75	1.4, 1.6
		RNA	0.65	6.5–7.5	4.20	0.9, 0.5
	E. coli B/r	DNA	1.28	2.2–2.7	0.62	3.5
		RNA	2.01	5.0–6.5	2.90	1.8

McFall (quoted by PARDEE AND PRESTIDGE[1]) calculated a DNA/RNA ratio for *E. coli* B/r which was twice that for strain B. No indication of the metabolic state of these cells was given, but this ratio, calculated from our data, is the same for logarithmic-phase cells of the two strains, and its value for resting cells of strain B/r is half that for similar cells of strain B.

Our results show that there is no significant difference in the concentration of DNA in logarithmic-phase cells of strains B and B/r grown in nutrient broth, while the difference in the amount of DNA contained in the resting cells of the two strains is small. It is just "significant" at the $P = 0.05$ level but doubt is thrown on the significance of this result by our lack of knowledge of the true distribution of values within a group, nor is it known whether the two strains were equally variable from batch to batch. The lethal effect of radiations on cells of these strains is markedly influenced by conditions after irradiation[16–19], but in general considerably higher doses of radiation are required to bring about the same lethal effect in strain B/r as in *E. coli* B. It is difficult to assign a numerical value to the ratios of radiosensitivities, as the shapes of the survival curves differ, but if we use as an arbitrary standard the doses required to reduce each surviving population to 10%, then the ratio of sensitivities may vary from one, for logarithmic-phase cells incubated on minimal medium at 37° after exposure to u.v. light[19], to 25, for resting cells irradiated with X-rays under oxygen and incubated at 19° on a nutrient medium[20]. Non-dividing cells of strain B are more resistant to u.v. light and X-rays than are dividing cells[18], but they contain perhaps slightly less, and certainly no more DNA. The radiosensitivities of cells of strain B/r differ more widely between these two physiological states, but the difference lies essentially in the point to which the exponential part of the survival curve extrapolates in a semi-logarithmic plot. The intercept which the extrapolated curve makes with the ordinate is usually called the "multiplicity", but we propose to refer to it as the "extrapolation number"[21]. A variation in extrapolation number, but not in the slope, may be remarked in the survival curves found by HOLLAENDER *et al.*[22] for cells of strain B/r grown in different ways. Similarly, the difference in sensitivity noted by BILLEN[2] for cells of B/r incubated before irradiation in a medium containing chloramphenicol is due to a difference in the extrapolation number. The extrapolation number for u.v.-irradiated *E. coli* strain B/r incubated on a minimal medium has been found to vary from one, for logarithmic-phase cells, to about 3 for stationary-phase cells, but there is no significant difference in the DNA contents of the cells in the two stages.

We conclude from this analysis that the radiosensitivity of strains B and B/r is not correlated, positively or negatively, with the DNA or RNA contents of cells of these strains.

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THE INCORPORATION OF RADIOCARBON FROM ATP AND AMINO ACID INTO NUCLEIC ACIDS OF *ESCHERICHIA COLI*

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SUMMARY

1. The incorporation of the adenylate moiety of labeled ATP into soluble cytoplasmic RNA of *E. coli* and the incorporation of [^{14}C]isoleucine into RNA and proteins has been observed.

2. Fractionation of *E. coli* preparations yielded one fraction which catalyzed incorporation of adenine nucleotide into the intranucleotide positions of RNA and another which catalyzed incorporation into the terminal position of RNA.

3. Some characteristics of the enzymes responsible for nucleotide-amino acid end-group labeling have been described. The adenylate incorporating activities have been resolved from the enzymes which activate amino acids and which probably catalyze the incorporation of amino acids into RNA.

4. The evidence presented suggests that incorporation of amino acids into *E. coli* proteins may involve analogous reactions to those described for mammalian systems.

INTRODUCTION

Studies on incorporation of radioactive amino acids into proteins of mammalian tissues have suggested that enzymes present in the non-particulate fraction of cytoplasm catalyze a carboxyl activation of amino acids by ATP[1-4] yielding enzyme-bound amino acyl adenylates, and transfer of amino acyl groups to low molecular weight ribonucleic acid molecules[5-9]. Subsequently, there is a transfer of the amino acyl moiety of such ribonucleic acid–amino acid complexes to ribonucleoprotein particles[7,10]. Although amino acid activating enzymes of the type found in mammalian tissues have also been found in bacteria[11,12], studies with *S. aureus*[13] and *A. faecalis*[14] demonstrated that radioactive amino acids may be incorporated into proteins of these species by a system containing a particulate fraction but apparently lacking amino acid-activating activity of the type found in mammalian tissues[14]. These observations suggest that incorporation of amino acids into bacterial proteins may occur by a mechanism different than that described for mammalian tissues, but do not exclude other pathways of amino acid incorporation. The present studies, carried out with

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Abbreviations: ATP, adenosine triphosphate; RNA, ribonucleic acid; Tris, tris(hydroxymethyl)aminomethane; CTP, cytidine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate. A.S., ammonium sulphate. RNase, ribonuclease; DN-ase, deoxyribonuclease.

preparations obtained from *E. coli* show that amino acid incorporation into ribonucleic acid and into protein can occur by a pathway which seems to be analogous to that found in animal tissues. The similarity in the nature and precursors of the nucleotide-amino acid end sequence in soluble cytoplasmic RNA, recently elucidated in mammalian preparations[15-17], are consistent with the sequence of reactions which appear to be associated with incorporation of amino acids into mammalian proteins.

Previous studies in this laboratory[8,9] with purified tryptophan-activating enzyme indicated that the amino acyl moiety of enzyme-bound amino acyl adenylate was enzymically transferred to soluble cytoplasmic RNA. Under conditions where considerable transfer of amino acid from enzyme-amino acyl adenylate complex to RNA was observed, the adenylate moiety was not incorporated into RNA. These results suggested that the adenylate portion of enzyme-bound amino acyl adenylate is not the direct precursor of terminal adenine nucleotide in RNA[15,17,18]. Similar studies with an amino acid-activating enzyme preparation from *Escherichia coli* also indicated that although enzyme-bound amino acids are transferred to *E. coli* RNA, transfer of enzyme-bound adenylic acid does not occur. Considerable incorporation of ^{14}C from labeled ATP into RNA present in the activating enzyme fraction was observed, however, and control incubations of activating enzymes in presence of [^{14}C]ATP and added RNA also resulted in incorporation of radioactivity into RNA. These results suggested the presence, in the amino-acid-activating enzyme fraction, of an activity responsible for incorporation of adenine nucleotide from ATP into RNA which did not involve intermediary participation of amino acyl adenylate. The incorporation of precursors of the nucleotide-amino acid end sequence into RNA of *E. coli* is described in this communication.

EXPERIMENTAL

Preparation of E. coli *enzyme fractions*

E. coli were grown for 16–18 h at 37° in a medium containing 0.1 % K_2HPO_4, 0.1 % KH_2PO_4, 0.1 % NaCl, 0.07 % $MgSO_4$, 0.4 % $(NH_4)_2SO_4$, 0.05 % Na citrate, 1 % N-Z CASE and 0.5 % glucose at pH 7.4. All succeeding operations were carried out at 4°. Cells were collected by centrifugation, washed once with cold distilled water, resuspended in 0.03 *M* Tris buffer, pH 7.0 (1 g wet wt. of cells/10 ml buffer), and 35-ml aliquots sonically disrupted for 7 min. The sonicate was centrifuged at 100,000 × *g* (average) for 2 h and the residue discarded. The pH of the supernatant was adjusted to pH 4.9 with 1 *M* acetic acid and the residue obtained by centrifugation at 30,000 × *g* for 30 min (pH 4.9-residue) was suspended in 0.1 *M* Tris buffer, pH 7.0. The resuspended residue was reprecipitated at pH 4.9 and resuspended in Tris buffer pH 7.0 two times. The final suspension contained 200 mg wet wt. of residue/ml buffer. The yield of pH 4.9-residue was 20 % (wet wt. basis) of initial weight of cells. The pH 4.9-residue suspension was diluted with an equal volume of 0.1 *M* Tris buffer, pH 7.0, 2 g of solid $(NH_4)_2SO_4$ were added/10 ml of suspension, and the solution stirred for 20 min. The precipitate was collected by centrifugation at 13,000 × *g* for 10 min, resuspended in a minimal amount of distilled water and dialyzed against running water for 5 h (20 % A.S. fraction). To the supernatant of the 20 % A.S. fraction, 2 additional g of solid $(NH_4)_2SO_4$ were added and the resulting residue was treated similarly to the 20 % A.S. fraction to yield the 40 % A.S. fraction.

The precipitation with $(NH_4)_2SO_4$ was continued and the 60% A.S. fraction was obtained.

Preparation of pH 4.9-residue nucleic acid

The pH 4.9-residue, recycled three times at pH values of 4.9 and 7.0 as described above, was diluted with 5 volumes of 0.02 *M* potassium phosphate buffer, pH 7.0 and extracted with water-saturated phenol at 4° (see ref. 19). After precipitation of potassium nucleate with 66% ethanol and dialysis against running water for 3 h, the preparation was lyophilized and resuspended in H_2O (20 mg/ml).

Methods

Incubations were carried out at 37.5° in 1 ml volumes in presence of the following components: KCl, 20 μmoles; $MgCl_2$, 10 μmoles; NaF, 1 μmole; Tris buffer, pH 8.0, 100 μmoles; phosphoenolpyruvic acid 10 μmoles; pyruvate kinase, 40 μg; nucleic acid, isolated from the pH 4.9-residue, 4 mg; CTP, 0.5 μmoles. Enzyme preparations were added as described. Radioactive precursors used were [^{14}C]ATP (1 μmole, 80,000 counts/min), [^{14}C]ADP (1 μmole, 90,000 counts/min), [^{14}C]AMP (1 μmole, 90,000 counts/min) or [^{14}C]isoleucine (0.07 μmole, 170,000 counts/min). Following the incubation period, 4 ml of cold 0.4 *M* $HClO_4$ were added and samples allowed to stand at 4° for 30 min. In experiments with labeled nucleotides, nucleic acids were isolated by two extractions of the washed acid-insoluble residue with 10% sodium chloride–0.01 *M* sodium Versenate at pH 7.0 for 30 min, followed by precipitation with ethanol and dialysis. Extractions in presence of Versene resulted in lower blanks in unincubated controls or in incubations with boiled enzyme preparations. When incubations were carried out with less than 2 mg of added nucleic acid, a corresponding amount was added as carrier immediately prior to addition of perchloric acid. In experiments with labeled amino acid, nucleic acid-amino acid complex was isolated by two extractions with 10% NaCl at 100° for 30 min[7]; extractions were carried out under acidic conditions, not neutralized as in the isolation of nucleotide-labeled nucleic acids. The residual proteins were isolated for radioactivity determinations as described[8].

Chromatography of nucleic acids hydrolyzed in 0.2 *M* NaOH at 80° for 3 h was carried out in ethanol–1 *M* NH_4 acetate, pH 7.5 (7:3); similar results were obtained with 2 other solvent systems. Chromatography of the acid-soluble nucleotides, following precipitation of perchloric acid as insoluble potassium salt, was also carried out with the solvent system described above.

RESULTS

The incorporation of [^{14}C]isoleucine into RNA and proteins of pH 4.9-residue obtained from *E. coli* sonicates, previously centrifuged at 100,000 × *g* for 45 min instead of 2 h as described above, is shown in Fig. 1. Incorporation of amino acid into RNA occurred first and loss of radioactivity was accompanied by incorporation of ^{14}C into proteins. Similar experiments with pH 4.9-residue as described above (2 h centrifugation at 100,000 × *g*) or with any of the subsequent fractions derived from it did not result in incorporation into protein; protein labeling described here is probably due to incomplete removal of particulates which are also sedimented at pH 4.9 and are capable of accepting activated amino acid from RNA-amino acid complex.

Incubation of the 100,000 × *g* supernatant (sonicate supernatant) obtained from sonically disrupted *E. coli* cells with $[^{14}C]$ATP (Table I) led to incorporation of radiocarbon into nucleic acids isolated: similarly, incubations with pH 4.9-residue or any of the $(NH_4)_2SO_4$ fractions derived from it also resulted in labeling of nucleic acid. The 20% A.S. fraction catalyzed primarily incorporation of ^{14}C from ATP

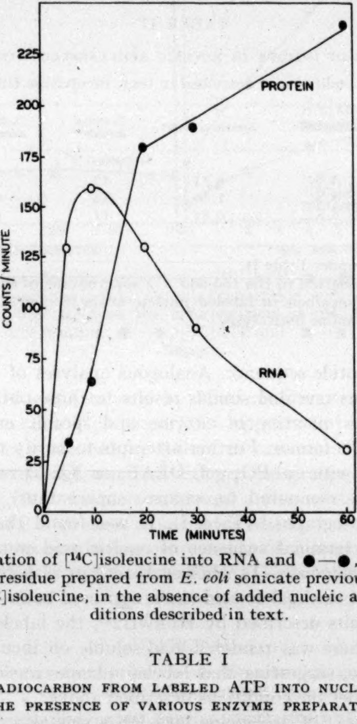

Fig. 1. ○—○, Incorporation of $[^{14}C]$isoleucine into RNA and ●—●, protein. Incubations were carried out with pH 4.9-residue prepared from *E. coli* sonicate previously centrifuged for 45 min at 100,000 × *g*, and $[^{14}C]$isoleucine, in the absence of added nucleic acid. Other incubation conditions described in text.

TABLE I

INCORPORATION OF RADIOCARBON FROM LABELED ATP INTO NUCLEIC ACIDS OF *E. coli* IN THE PRESENCE OF VARIOUS ENZYME PREPARATIONS

Incubations as described in text, contained $[^{14}C]$ATP and sonicate supernatant, 18 mg protein, pH 4.9-residue, 11 mg protein, 40% A.S. fraction, 4 mg protein, or Ca–PO_4 eluate, 0.3 mg protein. Incubations were carried out for 20 min.

Preparation	*Specific activity**	*% of initial activity*
Sonicate supernatant	0.16	100
pH 4.9-residue	0.18	85
40% A.S. fraction	1.67	54
Ca–PO_4 eluate	4.8	7

* Specific activity: mμmoles of $[^{14}C]$adenine nucleotide incorporated into the total nucleic acid fraction isolated, per mg of enzyme protein. Values are corrected for non-enzymic adsorption of ^{14}C observed in unincubated controls or in incubations with boiled enzyme preparations (0.05 mμmole/mg protein).

into intranucleotide (internal) sequences in nucleic acid; chromatographic analysis of alkaline hydrolysates of labeled nucleic acid revealed approx. 70 % as 3′-adenylic acid and 30 % as adenosine (Table II). The recovery of about 60 % of the radioactivity in adenosine from similar analysis of nucleic acids labeled with 40 % A.S. fractions indicated that this preparation catalyzed more extensively incorporation into the terminal adenine nucleotide sequence. Analogous analyses of nucleic acids labeled with 60 % A.S. fractions revealed similar results to those obtained with 40 % A.S. fractions; however, the quantity of enzyme and specific enzymic activity were considerably lower in the former. Further attempts to purify the enzyme present in the 40 % A.S. fraction with Ca–PO$_4$ gel, DEAE, or XE-64 resin, yielded a 30-fold purified preparation (as compared to sonicate supernatant) but resulted in 90 % loss of activity chromatographed (Table I). It was found that ability to catalyze incorporation into the terminal sequence of nucleic acid was lost gradually when 40 % A.S. fraction was stored at —15°; however, activity responsible for incorporation into the internal part of the nucleic acid chain appeared to be stable.

TABLE II

DISTRIBUTION OF ISOTOPE IN NUCLEIC ACID LABELED WITH $[^{14}\text{C}]$ATP

Incubation conditions as described in text; incubation time, 10 min.

Enzyme fraction	*Specific activity**	*% initial activity***	*% terminal adenylate****
20 % A.S.	1.73	17	30
40 % A.S.	1.80	54	61
60 % A.S.	0.63	11	60

* Specific activity (see under Table I).

** Per cent of activity observed in the 100,000 × *g* supernatant of sonicated *E. coli* cells.

*** Per cent of total radiocarbon in labeled nucleic acids recovered chromatographically as $[^{14}\text{C}]$adenosine following alkaline hydrolysis.

In contrast to results described by HURWITZ[20], the labeled product formed in incubations described here was rendered acid-soluble on incubation with alkali or RN-ase but not DN-ase, suggesting that labeled adenine nucletoide is incorporated into RNA of *E. coli* under the conditions described above.

The incorporation of $[^{14}\text{C}]$isoleucine into RNA was also catalyzed by sonicate supernatant, pH 4.9-residue, and the various ammonium sulfate fractions. Fig. 2 presents the time (A) and RNA-dependent (B) incorporation of ^{14}C from ATP and isoleucine in presence of 40 % A.S. fraction and supernatant obtained after treatment of the 40 % A.S. fraction with 2 % protamine sulfate (Protamine Super). Considerable incorporation of $[^{14}\text{C}]$adenine nucleotide occurred with 40 % A.S. fraction in absence of added RNA, due to the presence of nucleic acid in this enzyme fraction; addition of RNA to incubations resulted in a corresponding increase in incorporation of radiocarbon into RNA (2B). Treatment of 40 % A.S. fraction with protamine resulted in removal of approx. 98 % of the nucleic acid and 60 % of the protein from this preparation. A more marked RNA-dependent incorporation of $[^{14}\text{C}]$ATP into RNA was observed in incubations with Protamine Super. The effect of added RNA on incorporation of $[^{14}\text{C}]$isoleucine is also presented in Fig 2B. Although incorporation

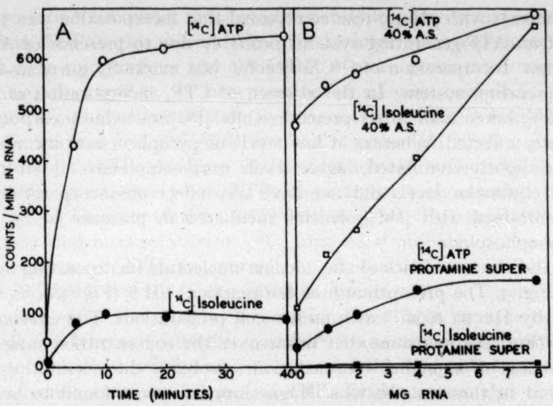

Fig. 2. A. ○—○, time-dependent incorporation of [^{14}C]ATP; ●—●, [^{14}C]isoleucine into RNA. Incubation conditions as described in text; enzyme preparation, 40 % A.S. fraction, 4 mg protein. B. ○—○, the effect of added RNA on the incorporation of [^{14}C]ATP and, □—□, [^{14}C]isoleucine into RNA; 40 % A.S. fraction; ●—●, [^{14}C]ATP and ■—■, [^{14}C]isoleucine; Protamine Super.

of [^{14}C]ATP into RNA was catalyzed by Protamine Super, incorporation of [^{14}C]-isoleucine was not detected. This preparation appears to be devoid of amino acid-activating enzymes, as evidenced by failure to observe an amino acid dependent ATP-pyrophosphate exchange reaction with several groups of amino acids. On the other hand, the 40 % A.S. fraction catalyzed amino acid dependent exchange of pyrophosphate and ATP with most amino acids tested.

The effect of various incubation components on incorporation of [^{14}C]ATP and [^{14}C]isoleucine into RNA is presented in Table III. Omission of an ATP-generating system or of RNA resulted in a slight decrease (20 %) in incorporation of [^{14}C]ATP;

TABLE III

EFFECT OF VARIOUS INCUBATION COMPONENTS ON THE INCORPORATION OF [^{14}C]ATP AND [^{14}C]ISOLEUCINE INTO RNA

Incubation conditions as described in text. Enzyme preparation, 40 % A.S. fraction; incubation time, 10 min.

Incubation mixture	*[^{14}C]ATP**	*[^{14}C]isoleucine***
Complete system	1.64	134
Complete-ATP-generating system	1.31	22
Complete-RNA	1.31	58
Complete-CTP	1.18	125
Complete + P–P (2 μmoles)	1.83	—
Complete + P–P (25 μmoles)	0.04	20
Complete + PO_4 (25 μmoles)	1.60	120

* Incubations with radioactive ATP. Values are expressed as specific activity (see under Table I).

** Incubations with radioactive L-isoleucine. Values are expressed as total counts/min incorporated into RNA.

similar experiments with pH 4.9-residue revealed that incorporation was 50% lower in absence of an ATP-generating system; probably due to presence of ATP-ase in this preparation. Incorporation of [^{14}C]isoleucine was markedly lower in absence of an energy generating system. In the absence of CTP, incorporation of [^{14}C]ATP was about 30% lower than in its presence while [^{14}C]isoleucine incorporation was not significantly affected. Whereas at low levels of pyrophosphate incorporation of [^{14}C]ATP was slightly stimulated, higher levels were completely inhibitory; monophosphate at equimolar levels did not have any effect on incorporation. Similar results were obtained with [^{14}C]isoleucine incubated in presence of 25 µmoles of pyro- and monophosphate.

Some of the characteristics of the adenine nucleotide incorporating system are presented in Fig. 3. The pH optimum of the reaction, pH 8 (Fig. 3A), is similar to that reported by HECHT *et al.*[17] with ascites cell preparations. The enzyme concentration curve (Fig. 3B) was somewhat linear over the concentration range of 4 mg of protein leveling off at higher concentrations, probably due to limiting amounts of RNA present in these incubations. Magnesium ions were found to be essential (Fig. 3C); manganese at equimolar concentrations could replace Mg^{++} and addition of 10 µmoles of Mn^{++} to incubation mixtures containing Mg^{++} resulted in 35% stimulation of incorporation. Radioactivity from labeled ADP as well as ATP was

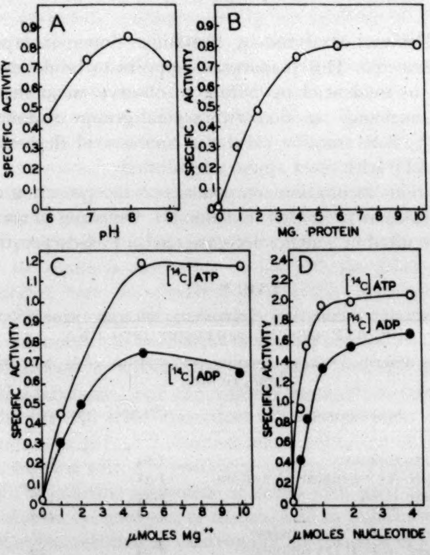

Fig. 3. Characteristics of the adenine nucleotide incorporation system. Incubation conditions as described in text; enzyme preparation, 40% A.S. fraction; incubation time, 10 min. A. Effect of pH on the incorporation of [^{14}C]ATP into RNA. B. Effect of enzyme concentration on the incorporation of [^{14}C]ATP into RNA. C. ○—○, effect of Mg^{++} on the incorporation of [^{14}C]ATP; ●—●, [^{14}C]ADP into RNA (PEP and Py-kinase omitted). D. ○—○, effect of labeled substrate concentration on the incorporation of [^{14}C]ATP and ●—●, [^{14}C]ADP into RNA (PEP and Py-kinase omitted).

incorporated into RNA under these conditions; ATP, however, appeared to be more effective than ADP in this process (Fig. 3D). In subsequent experiments with more purified enzyme preparations and [^{14}C]ADP, a more pronounced requirement for an ATP-generating system in incorporation af adenine nucleotide was evident. Chromatographic analysis of alkaline hydrolysates of [^{14}C]RNA labeled in presence of 40% A.S. fraction and [^{14}C]ADP revelead a pattern of labeling similar to that observed with [^{14}C]ATP. Analyses of the acid-soluble nucleotides in incubations with [^{14}C]ADP, in absence of an ATP-generating system, revealed that approximately 65% had been converted to ATP within 2 min. These and other results obtained suggest that incorporation of [^{14}C] from ADP probably occurs via the intermediary formation of ATP. In contrast to observations made with labeled ATP and ADP, [^{14}C]AMP was not incorporated in absence of an ATP-generating system.

Preincubation of the enzyme preparations and RNA to remove enzymically the terminal cytidylic and adenylic moieties[16,17] was unsuccessful, probably due to the presence of nuclease activity which rendered RNA incapable of accepting labeled adenine nucleotide even in presence of CTP. Evidence was also obtained which indicated that preincubation of enzyme for 10 min, in absence of RNA, resulted in marked loss of activity. Incorporation of [^{14}C]isoleucine into "preincubated" RNA was considerably lower than that described above in Table III, however, addition of CTP resulted in 30% stimulation of incorporation under these conditions.

DISCUSSION

Low molecular weight cytoplasmic RNA appears to be involved in incorporation of amino acids into proteins. The formation of RNA-amino acid complex from free amino acids and ATP[5,6,8,9,15,21,22], or from synthetic amino acyl adenylates[8,9], and the transfer of amino acid from RNA to microsomal proteins[7,10] are consistent with this suggestion. The observation presented here, that uptake of [^{14}C]-isoleucine into protein is preceded by incorporation into and release of radioactivity from RNA, is also consistent with the role of RNA as a participant in amino acid incorporation. This RNA appears to contain a specific nucleotide end-sequence consisting of cytosine nucleotides and a terminal adenine nucleotide[16,17,23,24]. Evidence has been presented which suggests that amino acid occurs bound to the 3′ (or 2′)-hydroxyl group of the terminal adenylate moiety of RNA[15,17,18]. The incorporation of cytidine and adenine nucleotides into the terminal sequence of RNA has been reported[17,23-26] and it has been shown that these nucleotide end-units are required for binding of amino acids to RNA[17].

HERBERT[26] has reported the presence of two activities in pigeon liver responsible for incorporation of adenine nucleotides into RNA. One of these, present in the nuclear fraction, catalyzes incorporation into the interior of the RNA molecule; the other, present in cytoplasm, catalyzes incorporation into the end-group position. The presence of separate enzymes which catalyze intranucleotide incorporation of adenine nucleotide[25,27] and of cytidine nucleotide[28,29] from their respective triphosphates into RNA has been reported. The results presented here indicate that the supernatant obtained by high speed centrifugation of sonically-disrupted *E. coli* cells contains enzymes which catalyze amino acid activation, incorporation of amino acids into RNA and protein, and incorporation of the adenylate moiety of ATP into interior and end-group positions of RNA. A partial resolution of these activities has been obtained. The evidence obtained suggests that, as in the case with mammalian preparations, terminal incorporation of cytidine and adenine nucleotides may be necessary for binding of activated amino acids to RNA in *E. coli*. Unfortunately, due to the presence of nuclease in the purified enzyme preparations, it has not yet been possible to prepare active RNA in which the terminal nucleotide sequence has been removed. Attempts to prepare stable *E. coli* preparations, free of interfering enzymes such as nuclease and myokinase, are in progress.

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DYNAMIC ASPECTS OF THE NUCLEOTIDE POOL OF BREWER'S YEAST DURING GROWTH

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SUMMARY

Chromatography of ethanolic and aqueous trichloracetic acid extracts prepared successively from a particular strain of yeast at various stages of growth revealed, in addition to nucleotides already recognised in other yeasts, a number of new compounds of this nature including derivatives of uridine diphosphate, various nucleotide-peptide anhydrides which may be intermediates in protein synthesis, and polynucleotides. During the onset of growth of the yeast the pool of alcohol-soluble nucleotides decreased, while the reservoir of nucleotides soluble in trichloroacetic acid increased; but later a steady state was attained. The consequent hypothesis that the alcohol-soluble compounds are precursors of the nucleotides soluble in trichloracetic acid, which in turn are converted into insoluble compounds, was supported by the results of experiments in which the uptake and subsequent metabolism of [^{14}C]adenine and uracil were examined. Entry of [^{14}C]uracil into a nucleotide-peptide anhydride was observed.

INTRODUCTION

Recent investigations[1,2] have shown that yeasts contain nucleotide pools analogous to the amino-acid pools detected earlier[3,4]. Furthermore[5], study of the incorporation of [^{14}C]purines into the nucleic acids of *Candida utilis* have shown that the adenine of the soluble nucleotides was labelled far more extensively than that of the nucleic acids, implying that the nucleotides have a greater turnover rate than the nucleic acids. Both added purines and nucleotides are ultimately incorporated into the nucleic acid of *C. utilis*, the acid-soluble nucleotides forming direct precursors of nucleic acid[6].

In order to gain further insight into the synthesis of nucleic acids and proteins in yeasts, in particular those used in brewing, an investigation of the nucleotide pool of a selected strain during aerobic growth has been made.

Abbreviations: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; AMP, adenosine-5′-monophosphate; ADP, adenosine-5′-diphosphate; ATP, adenosine-5′-triphosphate; GMP, GDP and GTP, guanosine-5′-mono-, di-, and triphosphate; UMP, UDP, and UTP, uridine-5′-mono-, di-, and triphosphate; CMP, CDP, and CTP, cytidine-5′-mono-, di-, and triphosphate; UDPAG, uridine diphosphate acetylglucosamine; UDPG, uridine diphosphate glucose; TCA, trichloroacetic acid.

MATERIAL AND METHODS

Culture of yeast

Saccharomyces cerevisiae (No. 240, British National Collection of Yeast Cultures) was grown in shake culture in sterile hopped wort (sp. gr., 1.040) for 48 h and was then centrifuged off and washed with water as quickly as possible (20 min). An aliquot portion was removed for extraction and analysis as described below and further samples (6 g fresh weight) were weighed into separate batches (250 ml) of the same brewers' wort in Roux bottles and growth was allowed to proceed with shaking at 24°. The individual crops of yeast were rapidly removed after various time intervals by filtration on kieselguhr pads[7]. It is relevant to note that in earlier investigations[8] the yeast was centrifuged off as rapidly as possible, but comparison of ion-exchange chromatograms of the extracted nucleotides (see below) with those prepared from extracts of filtered yeast showed that some decomposition of nucleotides, particularly adenosine triphosphate, was incurred in the earlier procedure.

Extraction of nucleotides from yeast

Each yeast crop was killed immediately by admixture with powdered carbon dioxide and treated with ethanol (1 ml/g yeast); the mixture was then allowed to come to room temperature and was then warmed for 3 min at about 50° to complete extraction of nucleotides, after which the yeast residue was filtered off. This residue was warmed twice more with 50 % ethanol (2 ml/g original yeast) and the ethanolic extracts were combined and stored at 0° prior to analysis.

Each yeast residue was dried by successively washing with ethanol, ethanol–ether mixture (1:1, v/v) and ether and stored at 0° prior to extraction with 5 % TCA. The TCA was cooled to 0°, mixed with the yeast residue (1 ml/g original yeast) and the suspension frozen and kept overnight at —15°. After thawing, the residue was immediately filtered off and the bulk of the TCA rapidly extracted from the filtrate by shaking (3 × 0.5 vol), the remaining TCA being removed by continuous extraction with ether for 16 h. The aqueous solution was stored at 0° in the presence of a few drops of chloroform until required for analysis.

Chromatographic separation of nucleotides

Aliquot portions of the ethanolic extract corresponding to 4 g of original yeast at each growth stage were loaded individually on to Dowex 1-X4 (formate) columns (20 cm × 1.77 cm^2) and the nucleotides eluted successively in a formic acid–ammonium formate gradient as described by GILBERT AND YEMM[7], the eluate being collected in 5 ml fractions and the u.v. light absorption at 260 mμ of the fractions taken as a measure of their nucleotide content.

Aliquot portions of the TCA extracts corresponding to 18 g of original yeast were similarly chromatographed on Dowex 1-X4 (chloride) columns as described by PONTIS AND BLUMSON[9].

Identification of nucleotides

The nucleotides were provisionally identified by comparison of their positions on the ion-exchange chromatograms and their u.v. light absorption spectra at various pH values with those of authentic nucleotides. In this connection, however, it was frequently impossible to measure complete absorption spectra directly on the eluates from the ion-exchange columns, particularly the solutions containing formate ions, which themselves absorb u.v. light strongly at wavelengths less than 245 mμ. The eluates obtained from the Dowex chloride columns did not suffer from this disadvantage but, it was on the other hand often not possible to measure their u.v. absorption in alkali owing to their becoming opaque.

Further characterization of the nucleotides was effected by (a) electrophoresis on paper using acetate buffer solution at pH 4.0 and a voltage gradient of 10–20 V/cm, (b) chromatography on paper in the solvent system II of KIRBY[10] and the isopropanol–hydrochloric acid solvent of WYATT[11] and (c) hydrolysis to the constituent purine base or pyrimidine nucleotide by means of 6 *N* hydrochloric acid at 100°. The purine bases and pyrimidine nucleotides thus formed were themselves identified by two-dimensional chromatography on paper using solvent systems composed of *n*-butanol–acetic acid–water (4:1:1, v/v) and *n*-butanol–ethanol–2 *N* ammonia (20:2:5, v/v) respectively[12]. The solvent composed of isopropanol and hydrochloric acid[11] was also employed.

The nucleotides and derived bases were detected on paper chromatograms and electropherograms by means of their absorption of u.v. light at 254 mμ.

Incorporation of labelled purines and pyrimidines

[8-^{14}C]adenine and [2-^{14}C]uracil were employed separately at the rate of 0.4 μmole/ml of wort. Yeast (0.5 g) was inoculated into wort (50 ml) containing the labelled base and grown up aerobically for 30 h in shake-culture at 24°. Aliquot portions (3 ml) of the suspension were removed from time to time; the yeast was removed from each by filtration through a thin kieselguhr pad and washed twice with distilled water and the mixture of yeast and kieselguhr was placed immediately in a mixture of 60 % ethanol and solid carbon dioxide. The whole procedure of collection, washing and inactivation of the yeast took about 1 min. Further extraction of the yeast with aqueous ethanol was effected as before and the whole extract was evaporated in the cold to a small volume, which was then made up to 10 ml. The yeast debris was extracted with aqueous TCA as before and the extract brought to 10 ml.

In further experiments labelled adenine or uracil was added to the yeast at different stages of growth using 20 ml of wort, 0.25 g of yeast and 4 μmole of labelled uracil or 10 μmole of labelled adenine.

Measurement of radioactivity

For measurement of radioactivity the growth medium and corresponding yeast extracts (0.5–1.0 ml) were individually mixed with cellulose powder, which was dried, combusted and the disintegrations of the carbon dioxide formed counted in a gas counter. Chromatograms and electropherograms of medium and extracts were scanned for radioactive components, using an end-window counter and autoradiographs prepared using Kodak X-ray no-screen film and a contact time between chromatogram and film of 10–20 days.

RESULTS AND DISCUSSION

The nucleotide content of the yeast at various times during aerobic growth is shown diagrammatically in Fig. 1, (a) and (b), (a) referring to the ethanolic extract and

(b) to the TCA extract. Incidentally, the advantage of employing the dual extraction procedure rather than direct extraction of the total nucleotides in aqueous TCA or perchloric acid as commonly carried out, is manifest, because the ethanolic solvent is more selective and the interpretation of the ion-exchange chromatograms is therefore simplified.

The ethanolic extracts of the yeast (Fig. 1 (a)) contained AMP, ADP, ATP, GMP, GDP, GTP, UMP, UDP and UTP as previously observed by SCHMITZ[1] and by AYENGAR *et al.*[13], together with CMP, DPN, TPN and various other substances among which were provisionally identified UDPAG and UDPG. It is apparent that, as in *C. utilis*[7], cytidine diphosphate and cytidine triphosphate must be present in relatively small concentration in the ethanol extracts, as no discrete peaks due to them were discerned. The previous observation[8] that the bulk of the pyridine nucleotide in the initial yeast was TPN, was not borne out in the present investigation and the requisite conditions for obtaining only TPN in the culture were not found again.

Only small amounts of nucleotide derivatives reactive towards hydroxylamine[14] as detected by HARRIS *et al.*[8] were recovered from Dowex 1 (formate) columns, because they are not adsorbed as they are when Dowex 1 (chloride) is used (*cf.* DAVIES AND HARRIS[12]).

The TCA extracts (Fig. 1 (b)) contained a number of nucleotides, none of which was identified with any known compound. Several of the earlier peaks however had

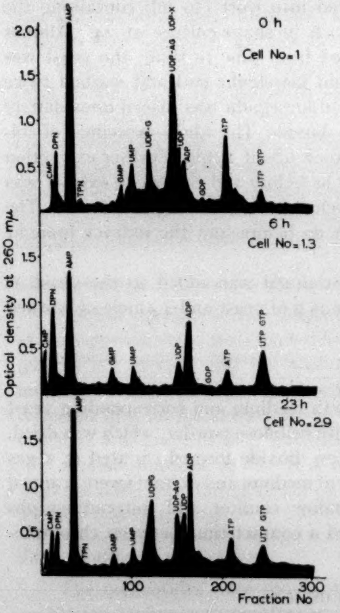


Fig. 1 (a). Ethanol-soluble nucleotide content of yeast at various stages of growth (for details see text).

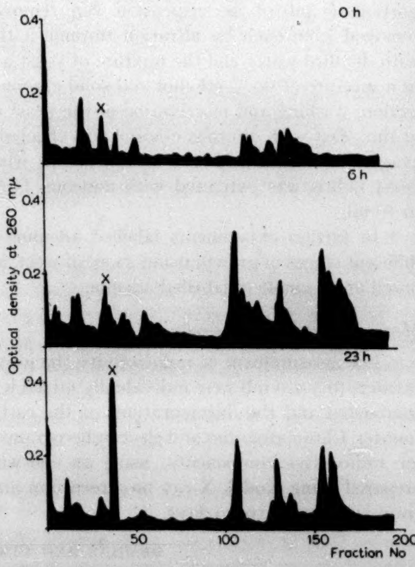


Fig. 1 (b). TCA-soluble nucleotide content of yeast at various stages of growth (For details see text).

u.v. absorption spectra like that of cytosine but one of the major constituents (X in Fig. 1(b)) had a spectrum like that of uridine. This component is probably identical with the substance 17 noted by HARRIS *et al.*[8]. The large peaks eluted later and particularly prominent in the 6 and 23 h samples were, on the other hand, complex, because on electrophoresis the bulk of u.v. absorbing material migrated towards the anode only as a diffuse band, and on hydrolysis with hydrochloric acid it yielded a mixture of adenine, guanine, uridylic acid and cytidylic acid. Moreover, in many cases the original peak substances contained not only negatively-charged nucleotides but also nucleotide-peptide anhydrides migrating towards the cathode[8,12], these being present because the TCA extracts were chromatographed on the chloride form of Dowex-1.

The nucleotide pattern of the yeast extracts after 6 h development of the cells was of particular interest, because it represents an early stage in the onset of growth. The amounts per cell of the majority of the alcohol-soluble nucleotides, with the exception of DPN, ADP and the UTP-GTP peak, had undergone a considerable reduction as observed earlier[8]. In view of the well-established role of ATP in energy transfers associated with synthetic processes in the cells, its depletion and the accompanying increase in ADP concentration may well reflect the demands of the onset of these synthetic processes. The decrease observed in other nucleotides, *e.g.*, UDPAG, also probably indicates the draining off of raw materials for the synthesis of proteins, nucleic acids and cell-wall components[15–17] at this stage of the life cycle of the yeast. Correspondingly, a large increase both in amount and diversification of the nucleotides extracted by TCA was observed. The later samples of yeast contained nucleotide pools which resembled more closely the original material.

From the above data it appeared possible that the nucleotides soluble in TCA were formed wholly or in part from the alcohol-soluble compounds. An attempt to gain information on this point was made using $[^{14}\text{C}]$adenine, uracil and thymine. It was shown in preliminary trials that the two former bases were taken up by the strain of yeast used. In the present experiments nearly 80 % of the added radioactivity in adenine was taken up by the yeast in 30 h and the only radioactive compound apart from adenine detected in the medium was a trace of adenosine. The progress of uptake of radioactivity is shown in Fig. 2 and agrees well with the results previously obtained by HARRIS AND PARSONS[18] using non-labelled adenine. The distribution of the absorbed radioactivity between ethanol-soluble, TCA-soluble, and insoluble material within the cell is given also in Fig. 2 on the basis of percentage of absorbed radioactivity, while the distribution of activity in these fractions in the total culture and in unit weight of cells is shown in Fig. 3. It is apparent that the

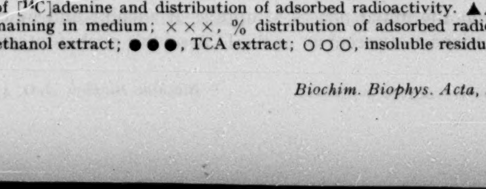


Fig. 2. Uptake of $[^{14}\text{C}]$adenine and distribution of adsorbed radioactivity. ▲▲▲, % of added radioactivity remaining in medium; × × ×, % distribution of adsorbed radioactivity between ethanol extract; ●●●, TCA extract; ○○○, insoluble residue.

radioactivity of all fractions increased from the time of addition, a large proportion of the absorbed activity initially entering ethanol-soluble material. After 1 h the relative activity of this fraction (Fig. 2) then declined, and that of the TCA extract increased, during a period (1–4 h) in which little relative increase of activity in insoluble material took place, although the absolute values (Fig. 3) were of course continuing to increase throughout. During the logarithmic phase of growth (4–24 h) the relative activity of the insoluble material continued to increase, while that of the TCA-soluble fraction was steady and that of the alcohol-soluble fraction fell, presumably because a steady rate of turnover of the acid-soluble material occurred. In the later phases, synthesis of insoluble material continued at the expense of both alcohol-soluble and acid-soluble fractions.

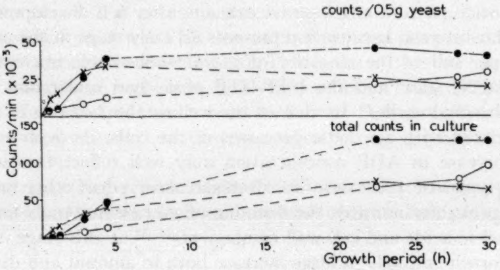


Fig. 3. × × ×, radioactivity present in ethanol extract; ● ● ●, TCA extract; ○ ○ ○, insoluble residue resulting from uptake of [^{14}C]adenine.

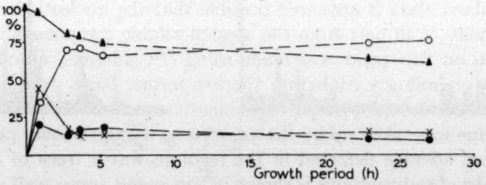


Fig. 4. Uptake of [^{14}C]uracil and distribution of adsorbed radioactivity. ▲▲▲, % of added radioactivity remaining in medium; × × ×, % distribution of adsorbed radioactivity between ethanol extract; ● ● ●, TCA extract; ○ ○ ○, insoluble residue.

The results for uracil were different in that a much smaller proportion of the added radioactivity was ingested by the yeast (Fig. 4) and that of the ingested base, a much higher proportion, promptly appeared in the insoluble and TCA-soluble fractions than was the case with adenine. Only in the first hour was the proportion soluble in ethanol greater than that in the other fractions, and thereafter the bulk of the radioactivity ingested was found in insoluble material (*cf.* Fig. 5).

Using labelled thymine of the same level of radioactivity as the other bases, only 0.5 % of the added thymine entered the yeast after 24 h and it therefore appears that the yeast has an impediment to the entry of this pyrimidine.

It is apparent that the above results for adenine and uracil support the earlier suggestion that in the initial phases of uptake of adenine and uracil the alcohol-soluble pool functions as a precursor of the TCA-soluble pool of intermediates.

Confirmation and extension of this result was obtained by adding labelled adenine and uracil to the yeast at different times early in development and comparing the uptake of these bases into the various fractions at these different times. The results are shown in Fig. 6 (a) and (b) from which it may be seen that the relative rate of uptake of labelled adenine into the TCA-soluble fraction was much slower than that into the alcohol-soluble materials during the first 2–4 h, but that later (4–8 h) it increased and subsequently predominated as growth proceeded. The results for uracil were similar, except that, as before, the relative incorporation into the acid-soluble fraction was much greater.

The transfer of activity from radioactive base to ethanol-soluble nucleotide, and in turn to TCA-soluble nucleotide, even during the very early period of growth, shows, not only that the ethanol-soluble materials act as precursors of the acid-soluble compounds, but also that a substantial synthesis of nucleotides (see below) takes place during the lag phase of growth.

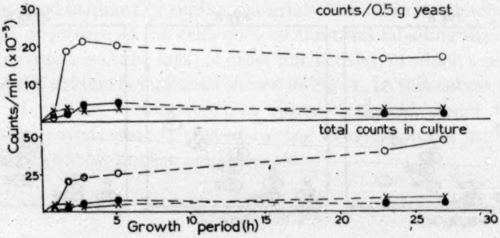
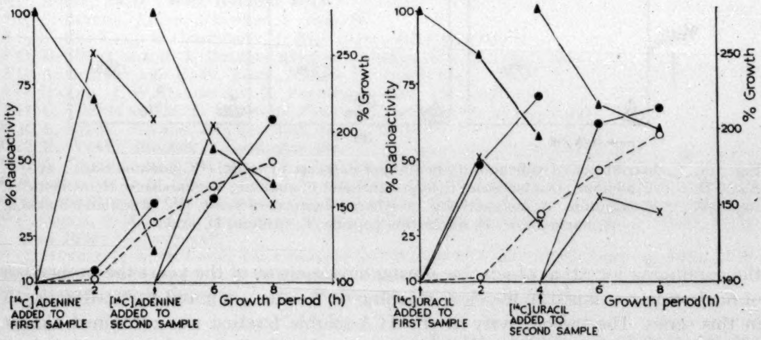


Fig. 5. × × ×, radioactivity present in ethanol extract; ● ● ●, TCA extract; ○ ○ ○, insoluble residue resulting from uptake of [^{14}C]uracil.



(a) (b)

Fig. 6 (a). Addition of [^{14}C]adenine to yeast at different growth stages. ○ ○ ○, Wt. of yeast as a % of wt. of original inoculum; ▲ ▲ ▲, % of added radioactivity remaining in medium; × × ×, % distribution of total radioactivity extracted between ethanol; ● ● ●, TCA extracts. Fig. 6 (b). Addition of [^{14}C]uracil to yeast at different growth stages. Details as for Fig. 6 (a).

In the case of adenine particularly, the results observed might have been due simply to the accumulation of large amounts of free adenine in the alcohol-soluble fraction in view of the rapid incorporation of this purine into the cell. However, it was remarkable that a large proportion of the absorbed adenine was converted into nucleotide very rapidly. This fact was established by two-dimensional chromatography of the alcohol-soluble fractions and estimation of the radioactivity present in the various zones. The results are shown in Fig. 7(a) from which it is seen that after only 1 ¼ h 46 % of the adenine was in the form of nucleotide, the remainder being in the form of the free base (8 %) and corresponding nucleoside (16 %) together with guanine[5] (7 %), guanosine (10 %) and, interestingly enough, the degradation product hypoxanthine (7 %). The compounds A and B (Fig. 7(a)) are presumably also degradation products, because they are more mobile than adenine in the chromatographic solvents used and are probably, therefore, of relatively low molecular weight. Despite the continuing ingestion of adenine during development of the yeast the proportion of nucleotide continued to increase, reaching 61 % after 4.5 h and 73 % after 22.5 h in this series. The radioactivity of the TCA-soluble fraction was contained almost entirely in nucleotides, the bulk of which, as already indicated for the fractions separated by ion-exchange chromatography, migrated towards the anode on electrophoresis at pH 4. This material gave on hydrolysis both labelled adenine and labelled guanine, as was anticipated from the earlier-observed conversion of the former to the latter purine[5].

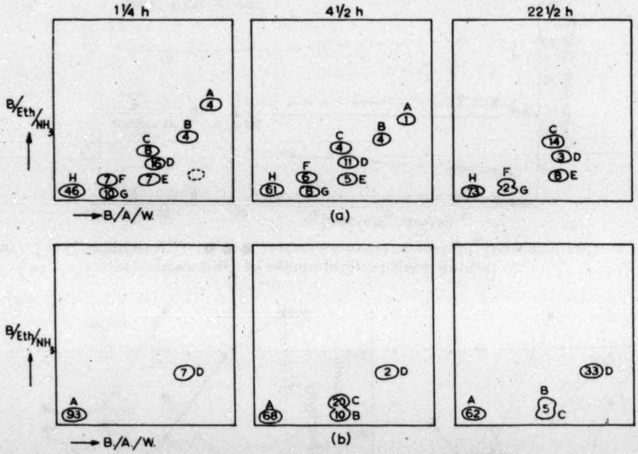


Fig. 7(a). % distribution of radioactivity in ethanol extracts of yeast, [^{14}C]adenine added at 0 h. A and B = ?; C, adenine; D, adenosine; E, hypoxanthine; F, guanine; G, guanosine; H, nucleotides.
Fig. 7(b). % distribution of radioactivity in ethanol extracts of yeast, [^{14}C]uracil added at 0 h. A, nucleotides; B, nucleotide-peptide; C, uridine; D, uracil.

As was expected from the more rapid rate of incorporation of uracil into the insoluble and TCA-soluble fractions, an even greater proportion (93 %) of uracil was located in nucleotides (Fig. 7(b)) and only 7 % remained free after 1 h. However, as uracil uptake proceeded throughout this trial, the proportion of chromatographically-immobile nucleotide decreased and after 4 h 20 % of the nucleoside uridine was observed, while after 23 h 33 % of the radioactivity in the ethanol-soluble fraction was present as uracil itself, presumably because the cell's requirements for this base for synthetic processes had by then been satisfied. Particularly noteworthy was the observation, made independently by measuring the combined radioactivity of the one zone containing uridine and nucleotide-peptides on electropherograms of the alcohol-soluble fractions, and substracting the activity of separated uridine as measured on the two-dimensional chromatograms (Fig. 8b), that as much as 10 % of the labelling was in the form of the nucleotide-peptides mentioned earlier. These nucleotide-peptides on alkaline degradation[12] yielded labelled uridine-5′-phosphate only. It appears that nucleotide-peptides derived from uridine may have a special significance in yeast, presumably as forerunners of proteins, and in the extracts examined they were found to exceed in quantity similar materials derived from adenine. Nevertheless, at the 4 h stage of growth of the cells 90 % of the total labelling derived from uracil in the ethanol extracts is in the form of other nucleotides, of which a major proportion must consist of the uridine compounds shown in Fig. 1. In this connection the specific incorporation of uridine derivatives into ribonucleic acid comes to mind, but it remains to be seen what part is played by the alcohol-soluble uridine compounds in the biosynthesis of this nucleic acid in yeast.

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DEOXYPENTOSE NUCLEIC ACIDS

XIV. THE DENATURATION OF DEOXYRIBONUCLEIC ACID IN AQUEOUS SOLUTION: PROPERTIES OF DNA DENATURED BY DILUTION

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SUMMARY

Sedimentation studies indicate that native DNA and DNA denatured by dilution with water below the critical concentration zone have similar sedimentation coefficients and sedimentation coefficient concentration dependence. This is similar to the findings for alkali and thermally denatured DNA.

Electron microscope investigations show that denaturation by dilution is attended by large morphological changes; however, it is thought that the observed molecular weight decrease is an artifact of the method used in the preparation of DNA samples for electron microscopy.

INTRODUCTION

It is generally accepted that the term denaturation[1-3] as applied to DNA,* should refer to the process involving the rupture of the specific hydrogen bonds that exist between the nitrogen bases in the native twin helix. A current problem of biological interest concerns the possibility of dissociating the constituent polynucleotide strands of the intact molecule. The first step of such a process would presumably involve the breakage of the hydrogen bonds holding the two strands in register. Studies involving the denaturation of DNA should therefore be pertinent to the above problem. Once denaturation has occurred the two polynucleotide strands are no longer bonded together. Because, however, of the plectonemic nature of the twin helix, the two strands cannot immediately dissociate. In the complete absence of any hydrogen bonding between the two strands, disentanglement by diffuse motion must take place before true dissociation can be said to have occurred. Calculations show that this dissociation is energetically feasible[4].

Experimental evidence reported for alkali[5], thermal[6] and low ionic strength denaturation[7] showed that denaturation is not accompanied by significant molecular weight change. It is therefore of interest to determine what factor is responsible for

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Abbreviation: DNA, deoxyribonucleic acid.

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the stability of the undissociated denatured state. If enough hydrogen bonds were present, in the denatured unit, then it could be imagined that diffusive motion would not lead to dissociation. Studies on denatured DNA[5,6] have led to the suggestion that under certain circumstances residual hydrogen bonds may exist. Two types of such residual hydrogen bonding may have to be considered. Some native hydrogen bonds having high bond strength may resist the denaturation process (evidence has already been reported[6,8] showing that hydrogen bond strength is heterogeneous in the native structure). Furthermore, it is possible that non-specific hydrogen bonds reform under some conditions of denaturation[5].

Previous communications from this laboratory have dealt with investigations of salt free DNA solutions and it was concluded that an essentially native structure existed at high DNA concentrations only. Dilution through a critical concentration zone was accompanied by denaturation. The critical denaturation zone was found to be $4–50 \cdot 10^{-5}\,M$ DNA for salt free solutions. Below this concentration zone the molecule was thought to be devoid of native hydrogen bonds. However, reconcentration of such a solution or the addition of salt was believed to result in the formation of a number of non-specific hydrogen bonds. In the present investigation some properties of DNA denatured by dilution to the critical concentration zone followed by reconcentration, will be compared with native calf thymus DNA.

EXPERIMENTAL

The preparations of calf thymus DNA used in this investigation have already been described[9]. Samples of DNA denatured by dilution were prepared in the following manner. A native stock solution of DNA ($3 \cdot 10^{-3}\,M$ in water) was diluted with water to $5 \cdot 10^{-5}\,M$, taking care to avoid ionic impurities. This solution was then freeze dried and redissolved to give $3 \cdot 10^{-3}\,M$ DNA in water. The process of denaturation in water, with or without subsequent freeze drying, results in an atomic extinction coefficient with respect to phosphorus concentration (measured in 0.1 M NaCl at room temperature) of 7340 ± 60, compared with the average value of 6660 for essentially native samples. All DNA concentrations were determined by phosphorus analysis on each stock solution. Sedimentation velocity experiments were made with a Spinco model E ultracentrifuge equipped with an u.v. absorption optical system. The experimental records were converted into plots of photographic density *versus* distance with an Analytrol photodensitometer. All the sedimentation experiments were made in 0.2 M NaCl at temperatures close to 25°, the sedimentation velocities were then corrected to water as solvent at 20°.

The electron microscope used was a Metalix 1190 fitted with the latest type of objective lens supplied by the manufacturer. Some of the later work was carried out with an improved electron gun. The converted instrument was equivalent in performance to the Philips EM-100 electron microscope. The technique described by HALL[10–12] was followed in the preparation of the samples for microscopy. Samples of native DNA were prepared by dilution of an aqueous stock solution to $2–5 \cdot 10^{-5}\,M$ DNA with a solution containing 0.1 M ammonium acetate and 0.05 M ammonium carbonate. The denatured material was prepared by dilution of a salt free stock solution to $2–5 \cdot 10^{-5}\,M$ DNA with water. The sprayed specimens were shadowed with platinum at shadow to height ratios between 5:1 and 10:1.

RESULTS AND DISCUSSION

Sedimentation

When a salt free solution of DNA is diluted below the critical concentration zone, denaturation[13] is accompanied by an increase in the effective charge and an increase in the resistance offered to motion of the kinetic unit. However, these changes were deduced for denatured DNA below the critical concentration and in the absence of salt. Reconcentration of such a solution, or the addition of salt, is thought to bring about the formation of non-specific hydrogen bonds and under these conditions the denatured kinetic unit would have quite different properties. For example, CAVALIERI, ROSOFF AND ROSENBERG[7] have shown that denatured DNA at high ionic strength has a decreased root-mean-square end-to-end distance (at constant molecular weight) as compared with a native sample.

Earlier investigations[14] showed that sedimentation velocity experiments on salt free DNA solutions yielded only a single boundary at DNA concentrations within the critical zone, although at these concentrations it is believed that native and denatured species coexist. It would be expected, from the discussion above, that the two species should exhibit different sedimentation properties, therefore further efforts have been made to resolve the two species by sedimentation.

An objection to the sedimentation experiments in salt free solution is that the critical concentration zone may not represent a region of coexistence of two species, but rather a change in property of either one. To overcome this objection it was necessary to compare the sedimentation properties of separate samples of both native and denatured DNA. This was carried out by a comparison of essentially native DNA (prepared by dilution of a salt free stock solution, at $3 \cdot 10^{-3}\,M$, to various DNA concentrations in the presence of salt to give 0.2 M NaCl) with DNA denatured by dilution, which was prepared by dilution of a similar stock solution to $5 \cdot 10^{-5}\,M$ DNA in the absence of salt followed by freeze drying and dissolution to $3 \cdot 10^{-3}\,M$ DNA. The various dilutions of the denatured material were then made by diluting in the presence of salt to give 0.2 M NaCl. The results of these experiments are shown in Fig. 1, where the variation of the sedimentation coefficient with concentration is given for both native DNA and DNA denatured by dilution. No significant difference in the sedimentation behaviour for the native and denatured material was found in these two preparations. A similar result (shown in Fig. 2) was found for a further DNA

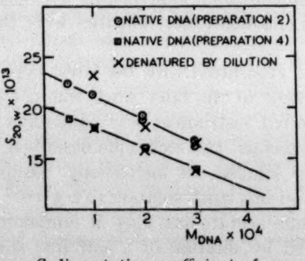

Fig. 1. Sedimentation coefficient of essentially native DNA and DNA denatured by dilution. All measurements made in 0.2 M NaCl at 25.0°.

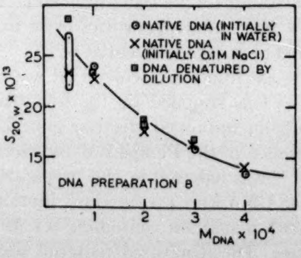

Fig. 2. Sedimentation coefficient of native DNA, essentially native DNA and DNA denatured by dilution.

preparation (see below). Mixtures of native and denatured DNA could not be resolved in further sedimentation experiments in 0.2 *M* NaCl; even at high DNA concentrations where differences in interaction would be expected, the two species sedimented together. The results shown in Figs. 1 and 2 also show that no significant difference in the sedimentation velocity concentration dependence exists between the native and denatured species of any one preparation. Although no differences in velocity were apparent in these experiments, the denatured material exhibited a more rapidly spreading boundary than native DNA.

No explanation can at present be advanced to explain the similarity in sedimentation properties between native DNA and DNA denatured by dilution. A similar problem has also arisen in the investigations concerned with alkali[5] and thermal[6] denaturation, where the native and denatured species also cannot be resolved by sedimentation. As has already been pointed out[6], it is difficult to put forward a mechanism whereby two species of similar molecular weight, but which differ by a factor of three in radius of gyration (a factor of 27 in molecular volume), can sediment at equal rates. In the authors' opinion this problem may ultimately be resolved by a consideration of possible differences in interaction between solvent and the native and denatured molecules respectively. In a discussion of this problem[6] the above type of interaction was assumed to be of equal magnitude for both DNA species.

Two further factors related to the above problem deserve attention. In contrast to native DNA the denatured species appears to have a high degree of flexibility. At zero ionic strength denatured DNA has a high charge and low mobility[13], indicating a highly extended molecule. On the other hand, at high ionic strengths denatured DNA has greatly reduced dimensions compared with the native unit[5–7]. The results at intermediate ionic strengths are quite compatible with these findings. It has been found, for example, that alkali denatured DNA exhibits a viscosity increase of about 8-fold on decreasing the ionic strength from 0.165 to 0.0055. (see ref. 5) and that this is followed by an increase of 2.3-fold in radius of gyration. EHRLICH AND DOTY[5] conclude that, at an ionic strength of $5.5 \cdot 10^{-3}$, the space filling properties of denatured DNA are about equal to that of the native structure. Viscosity studies on thermally denatured material also confirm that denatured DNA has a high degree of flexibility[6]. In view of the flexibility and relative stiffness of the denatured and native units respectively, it would be expected, accepting the observation that the two units have similar sedimentation velocities at high ionic strengths, that differences in this property would arise at lower salt concentrations. Preliminary investigations[15] concerned with sedimentation at low ionic strengths (but not at zero salt concentration) have already shown that resolution of the two species is possible; however, it is thought that this arises directly from the differences in charge rather than from the related shape change that occurs in the denatured unit.

Although the process of denaturation by dilution has been shown to occur over a critical concentration zone ($4\text{-}50 \cdot 10^{-5}$ *M* DNA), there is evidence which indicates that a small amount of denaturation accompanies dissolution of DNA in water at very much higher concentrations. An irreversible increase in atomic extinction coefficient (measured at 2590 Å in 0.1 *M* NaCl) of 1.4 % has been reported[9] on dissolving DNA in water to give a final concentration of $3 \cdot 10^{-3}$ *M*. Similarly, solutions of DNA in water at concentrations as high as $2\text{-}3 \cdot 10^{-3}$ *M* DNA suffer a small but significant irreversible decrease in viscosity (measured in 0.2 *M* NaCl), as compared with a sample of DNA never dissolved at low ionic strength[7]. In view of these findings it is pertinent to determine whether the small amount of denaturation inherent in the essentially native salt free samples used in this and earlier investigations (stock solutions of $3 \cdot 10^{-3}$ *M* DNA in water) is responsible for the similarity of sedimentation velocity between essentially native DNA and DNA denatured by dilution. An investigation of preparation 8 yielded information on this problem. This preparation had never been dissolved at low ionic strengths during preparation[9] and had therefore not suffered the small amount of denaturation discussed above. However, the precipitation of this preparation with ethanol was carried out at relatively low ionic strength (0.0014 *M* NaCl) and therefore the occluded salt impurity should be low. This preparation exhibited the typical critical concentration phenomenon[13,14] shown by the earlier salt free DNA preparations (precipitated at zero ionic strength). Preparation 8 can therefore be used to determine whether or not the small amount of denaturation which accompanies dissolution in water at high DNA concentrations has any bearing on the apparent similarity of sedimentation velocity of native DNA and DNA denatured by dilution to very much lower concentrations (below the critical zone). Fig. 2 gives a comparison of the sedimentation velocities shown by three solutions relevant to the present problem: (a) DNA initially dissolved in 0.2 *M* NaCl (having not suffered the small irreversible increase in $\varepsilon(P)$, (b) DNA initially dissolved in water at $3 \cdot 10^{-3}$ *M* DNA (having suffered the small irreversible change) and then diluted in the presence of salt to give 0.2 *M* NaCl, and (c) a solution of DNA diluted in water below the critical concentration, followed by freeze drying and redissolution in 0.2 *M* NaCl. It can be seen that no large difference in sedimentation velocity or sedimentation velocity concentration dependence is apparent in these three solutions. Therefore the small amount of denaturation that follows dissolution in water at high DNA concentrations cannot be held responsible for the similarity in sedimentation velocity between native DNA and DNA denatured by dilution in water below the critical concentration zone.

An interesting difference is to be noted between the sedimentation velocity concentration dependence shown in Figs. 1 and 2. Preparation 8 yields an increasing concentration dependence with decreasing concentration compared with the linear dependence exhibited by preparations 2 and 4. Although this cannot be related to the problem just discussed (two of the solutions shown in Fig. 2 having already been dissolved in water), a similar phenomenon was also found for preparation 7 (see ref. 9) which, however, was of even greater magnitude. Preparations 7 and 8 yielded a gel-like sediment on preliminary centrifugation (at 13,000 rev./min) of an aqueous stock solution ($3 \cdot 10^{-3}$ *M* DNA). This preliminary treatment of preparation 7 reduced substantially the curvature in the concentration dependence of the sedimentation velocity. No gel-like sediment was observed after preliminary centrifugation of stock solutions of preparations 2 and 4 (shown in Fig. 1). The results given in Figs. 1 and 2 were obtained after preliminary centrifugation of the stock solutions.

Electron microscopy

Visualization of single DNA molecules using the electron microscope[10-12] has made possible a direct comparison between native DNA and DNA denatured by dilution. The results of an investigation of native DNA confirm the previous findings of Hall[10-12]; however, the average molecular diameter was found to be 15 ± 8 Å,

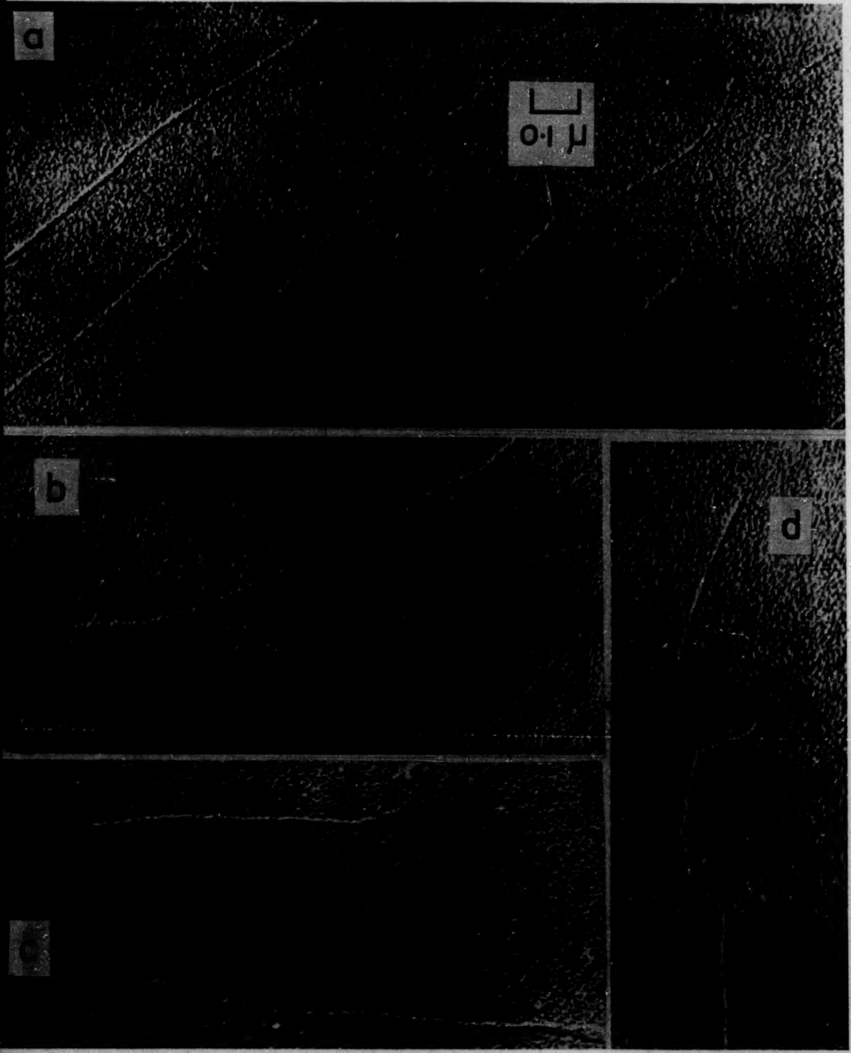

Fig. 3. Electron micrographs of essentially native DNA.

compared with the value of 20 Å given by HALL. Electron micrographs of the native material are shown in Fig. 3. From a study of many such fields it has become apparent that, although the initial DNA concentration was low, on drying, a longitudinal

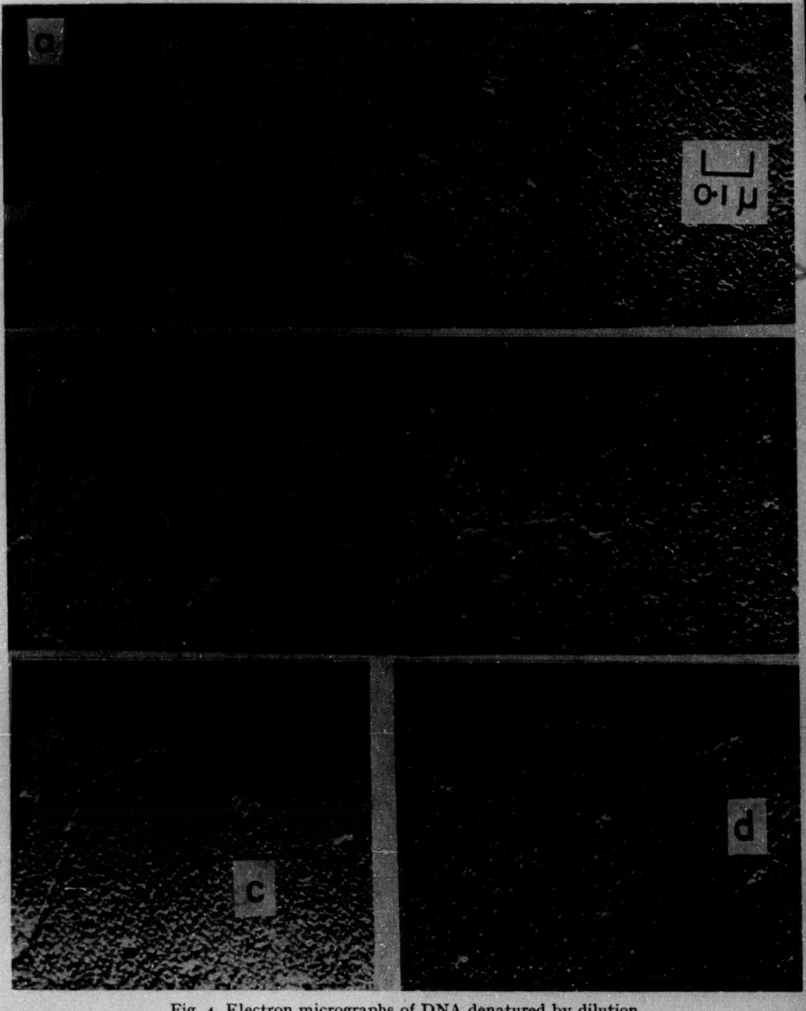

Fig. 4. Electron micrographs of DNA denatured by dilution.

aggregating tendency caused many of the molecules to lie together. This type of behaviour is shown in Fig. 3d. In addition a small amount of the native material would appear to have a molecular diameter smaller than that consistent with the diameter of the DNA double helix.

Before comparing the native material with denatured DNA, an interesting and perhaps important observation must be discussed. In agreement with HALL it is found that a small amount of the native DNA terminates in what appears to be flat patches (Fig. 3, a and c). It has been suggested that this phenomenon is to be associated with a small amount of denaturation[12]. However, electron microscope studies on DNA preparation 8 (a preparation never dissolved at low ionic strength) again yielded the characteristic flattened terminal patches and occasional patches along the native strand. Although these flat patches could be due to a small amount of denaturation inherent in calf thymus samples prepared by the detergent method[16], it is possible, in view of a somewhat similar observation made on calf thymus nucleoprotein[17], that this phenomenon may be a true feature of the molecular architecture of the chromosome.

Fig. 4 shows the fields obtained for DNA denatured by dilution below the critical concentration. A spectacular change from the rod-like material to collapsed or "melted out" DNA can be seen. The size of the patches indicates that coiling of the denatured strands has taken place. Two points of interest should be discussed. Firstly, Fig. 4 b, c, d, show remnants of rod-like material which have apparently resisted the denaturation process. It is to be noted that Fig. 4 is not typical in this respect; an average field would yield about one undenatured portion to every four fields examined. Secondly, in Fig. 4a, a completely denatured DNA field is shown and in this it will be noticed that the collapsed patches are too numerous to correspond to one patch for every native DNA molecule. This can be seen by comparing the number of collapsed units with the number of unimolecular strands shown in Fig. 3 (both figures represent samples with roughly equal two dimensional concentrations). That each of these collapsed units can only represent a portion of the original native molecules can also be seen from Fig. 4b,c,d, here presumably unimolecular strands are melting out into more than one collapsed unit. This observation is at variance with the constancy of molecular weight attending denaturation. It would therefore appear that the observed molecular weight decrease (found in the electron microscope studies) is an artifact of the method used to prepare the samples for microscopy. Support for this claim was found in further fields of denatured DNA, in which it was noticed that some of the collapsed units lay in lines reminiscent of the original native molecule. The collapsed units occurred at intervals of 1000–2000 Å, and were similar to those shown in Fig. 4 b and c, except that no interconnecting material was observable. The fact that in some instances the denatured patches lie in definite lines although unconnected, indicates that the denatured material has a larger molecular weight when in solution than the resulting specimens observed by electron microscopy. It is estimated that each patch corresponds to a molecular weight of $2\text{-}4 \cdot 10^5$ (assuming that a native molecule melts out into a denatured unit every 1000–2000 Å). As the denatured material dries on the mica slide each molecule will eventually be surrounded by a small amount of water and it is possible that the observed decrease in molecular weight could come about if some parts of each molecule were held more strongly to the mica surface during the final stages of drying. If this were so, then the forces accompanying the receding water phase could possibly bring about rupture of covalent bonds and hence a decrease in molecular weight. It has already been reported that, when a DNA solution, denatured by dilution followed by freeze drying and dissolution to $3 \cdot 10^{-3}\ M$, is precipitated from $1\ M$ NaCl with ethanol, a further irreversible change occurs and that this is accompanied by decreased molecular weight[18]. This is added evidence that the denatured material is rather susceptible to degradation on rapid changes from the dissolved to the solid state.

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DEOXYPENTOSE NUCLEIC ACIDS

XV. THE SEDIMENTATION OF CALF THYMUS DEOXYRIBONUCLEIC ACID IN 95% ETHANOL

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SUMMARY

The sedimentation properties of calf thymus DNA dissolved in ethanol have been studied and sedimentation coefficient distributions have been obtained. Evidence is presented for the collapsed state of the DNA molecule in ethanolic solution compared with aqueous solutions.

Abbreviation: DNA, deoxyribonucleic acid.

INTRODUCTION

The sedimentation coefficient of calf thymus DNA when measured in dilute sodium chloride solution depends only to a limited extent on the molecular weight of the sample[1,2]; this is exemplified by the observation of DOTY, MCGILL AND RICE[3] that for a series of samples of DNA of different molecular weights, the sedimentation coefficient (S°) for solutions in 0.2 M NaCl is proportional to $M^{0.37}$, where M is the molecular weight of the sample. Consequently such sedimentation experiments are of limited use for characterising the heterogeneity of DNA preparations. In principle sedimentation experiments would be more sensitive to molecular weight heterogeneity if they could be carried out under conditions such that the sedimentation coefficient depended more directly on the molecular weight. In this communication experiments will be described in which DNA was sedimented when dissolved in 95 % ethanol. For a variety of reasons (see below) it is believed that under these conditions the sedimentation coefficient depends to a much greater extent on the molecular weight of the DNA.

Consequently it is possible, by means of an analysis of the boundary spreading during sedimentation, to obtain estimates of the heterogeneity of DNA preparations which are more directly related to the molecular weight heterogeneity of the preparation, than are the results obtained on aqueous solutions.

The insensitivity of the sedimentation coefficient of DNA to the molecular weight of the sample when it is dissolved in 0.2 M NaCl is a consequence of the extended free-draining nature of the molecule. Theoretically the sedimentation coefficient of a free-draining random coil should be independent of the molecular weight[4]. The limited molecular weight dependence of the sedimentation coefficient of the DNA molecule in 0.2 M NaCl must therefore indicate that DNA is not behaving as a completely free-draining random coil under these conditions. The configuration of the DNA molecule in solution depends on the following factors: (a) the inter-nucleotide hydrogen bonds which confer considerable stiffness on the molecule, (b) the repulsions between the negative charges situated on the ionised phosphate groups of each nucleotide, and (c) the solvation of the molecule. In a "good" solvent a flexible molecule is extended allowing many solute–solvent interactions, and the free energy of the molecule is consequently reduced.

When dissolved in 0.2 M NaCl the above factors collectively lead to an extended free-draining structure for the DNA molecule. In order to carry out sedimentation measurements in a manner such that the sedimentation coefficient depends more directly on the molecular weight, this extended molecule must be collapsed giving rise to a non or partial free-draining molecule. GEIDUSCHEK AND GRAY[5] showed that considerable contraction occurred in the DNA molecule if it was dissolved in 95 % ethanol in the absence of traces of sodium chloride. Using light scattering measurements they observed that the radius of gyration of the DNA molecule and its molecular weight were 980 Å and $6.6 \cdot 10^6$ respectively when dissolved in 95 % ethanol, compared with 3000 Å and $7.7 \cdot 10^6$ when dissolved in 0.2 M NaCl. This contraction probably comes about by modification of the second and third factors mentioned above. There is no reason to suspect *a priori* that dissolution in ethanol will lead to the scission of internucleotide hydrogen bonds. However, in view of the conclusions of THOMAS[6], the prolonged dialysis of DNA against distilled water could conceivably lead to hydrogen bond scission, since he found that DNA was denatured in solutions of low ionic strength. Such denaturation is not likely to have occurred in the experiments described here because the dialyses were carried out at a concentration of DNA of 0.1 %, and INMAN AND JORDAN[7] have found that denaturation does not occur if the DNA is kept at a concentration above 0.007 % even in the total absence of added electrolyte. The results of GEIDUSCHEK AND GRAY support our contention on this point since they found that there was no increase in the u.v. absorption of ethanolic solutions of DNA compared with saline solutions such as would have occurred had the DNA been denatured.

Conductivity measurements show that the charge on the DNA molecule is much reduced when dissolved in 95 % ethanol as compared with water. This indicates that the effective number of charged phosphate groups is reduced. The equivalent conductivity of DNA in 95 % ethanol is of the order of one percent of the equivalent conductivity of DNA in water. Presumably this is a consequence of the lower dielectric constant of ethanol (24 as opposed to 82 for water) leading to greater ion pair formation between sodium ions and ionised phosphate groups. It is also reasonable to suppose that the solvation of the DNA molecule will be less in ethanol than in water because of the lower hydrogen bonding capacity of ethanol. Furthermore, if the hydration of DNA is closely connected with an ice-like arrangement of water molecules on its surface as suggested by JACOBSEN[8], it is unlikely that ethanol will solvate to the same extent as water.

EXPERIMENTAL

The calf thymus DNA was prepared by the method of KAY, SIMMONS AND DOUNCE[9] and its properties have been described previously[10].

Since the 95 % ethanol DNA solutions contained no involatile substances other than DNA, DNA concentrations were determined by weighing the amount of solid material in a weighed amount of solution. A weighed quantity of ethanolic DNA solution was placed in a desiccator over KOH pellets at atmospheric pressure. The DNA residue reached constant weight in about three weeks; this procedure was found to give reproducible results.

A pure azeotropic 95 % ethanol solution was prepared by refluxing redistilled spirits of wine over caustic potash for several hours, and distilling the product through a fractionating column. The middle third only of the distillate was subsequently used. Spirits of wine was used as a starting material because of the difficulties of removing traces of benzene from commercial absolute alcohol.

The dissolution of DNA in ethanol was carried out by the method of GEIDUSCHEK AND GRAY[5] at 0–4°. A sample of DNA dissolved in distilled water was freed from salt by dialysis against distilled water. The salt free aqueous DNA solution was then dialysed against a 25 % ethanol/water solution which was replaced with increasingly concentrated ethanol solutions up to 95 % azeotropic ethanol. The solution was finally dialysed twice against 95 % ethanol. All dialyses were carried out at 0–4° dynamically, by slowly oscillating about its vertical axis a sealed jar containing the dialysis bag and the outside solution.

Sedimentation experiments were carried out using a Spinco model E ultracentrifuge equipped with schlieren optical system. The schlieren pattern was produced by means of an inclined wire of bright 33 gauge Nichrome. Runs were carried out at 39,460 rev./min using a standard A analytical rotor and standard 4° sector 12 mm cells. Solution temperatures were determined by measuring the rotor temperature with a thermocouple before and after the run and taking a mean value. No correction was applied for the adiabatic cooling of the rotor. The photographic plates were measured in a Pye two-dimensional comparator reading to 0.01 mm. Sedimentation coefficients were corrected to a medium of the same viscosity as water at 20°; however, no correction was applied for the difference in density between water at 20° and 95 % ethanol. Graphs of refractive index gradient in the cell for boundary analysis, were obtained by tracing an enlarged projected image of the photographic plate onto graph paper. Areas were determined by planimetry of the tracings.

The partial specific volume of DNA in ethanol was determined by pycnometry. A graph of weight of solution against weight concentration of DNA was plotted, from which the partial specific volume term $(1 - \bar{V}\rho)$ was determined[11]. A value of 0.38_1 ml/g was obtained for the partial specific volume, $\bar{V}$.

RESULTS AND DISCUSSION

A typical ultracentrifuge photograph of the sedimentation of DNA in alcohol is shown in Fig. 1. It can be seen that a single rapidly sedimenting peak is obtained, which spreads very rapidly with time. In earlier experiments using shorter dialysis times it was often found that two peaks were observed in the ultracentrifuge (Fig. 2).

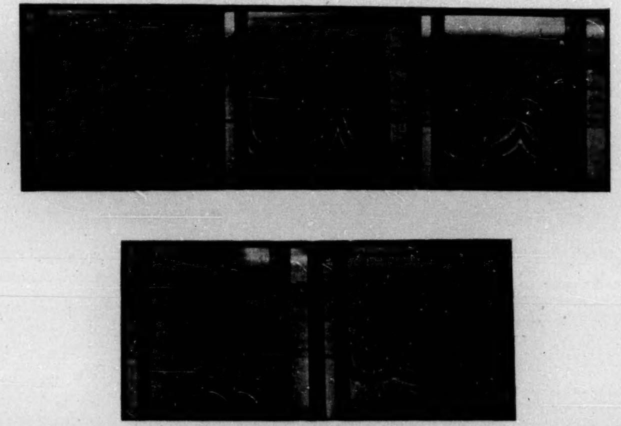

Fig. 1. Sedimentation of DNA in ethanol showing one peak (direction of sedimentation from right to left).

When the time of dialysis against distilled water was increased the smaller peak disappeared and symmetrical peaks of which Fig. 1 is typical were always obtained. The second smaller peak probably represents some form of DNA aggregate which arose because of a residual trace of sodium chloride in the solution. This conclusion is supported by the observation that the addition of 0.1 ml of ethanol saturated with

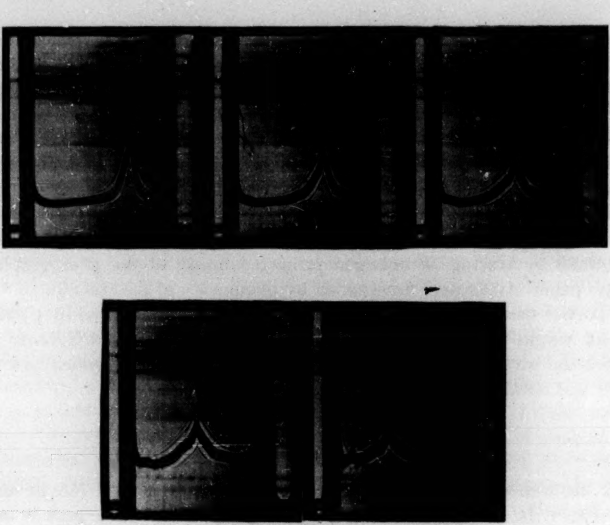

Fig. 2. Sedimentation of DNA in ethanol showing two peaks (direction of sedimentation from right to left).

sodium chloride to 1 ml of an ethanolic DNA solution causes immediate gelation of the solution.

Sedimentation experiments were carried out over a range of concentrations with both the single component and the two component ethanolic DNA solutions. The mean sedimentation coefficients for the various components were computed by measuring the rate of movement of the maxima of the refractive index gradient photographs. The results obtained are shown in Table I and Fig. 3.

TABLE I

SEDIMENTATION BEHAVIOUR OF DNA IN 95% ETHANOL WHEN TWO COMPONENTS ARE PRESENT

Total DNA concentration %	S_{20} *large peak S units*	S_{20} *small peak S units*
0.86	51.9	71.6
0.86	53.7	66.5
0.41	87.0	63.1
0.18	89.0	Only one peak detected
0.09	111.9	

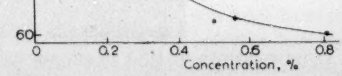

Fig. 3. The concentration dependence of the sedimentation coefficient of DNA in 95% ethanol when only one component is present.

From these data it can be seen that the larger of the two sedimentation peaks probably corresponds to the single peak observed when more prolonged dialysis was used to remove traces of sodium chloride. Restricting attention to the data for the single component solutions, (Fig. 3), it can be seen that there is very marked concentration dependence of the sedimentation coefficients and the individual values are very much greater than those observed for DNA dissolved in 0.2 *M* NaCl[11].

It would be of interest to obtain a value for the sedimentation coefficient at infinite dilution. Extrapolation of the graph in Fig. 3 is clearly not possible with any precision. The theories of FESSLER AND OGSTON[12] and of BURGERS[13] for the concentration dependence of sedimentation coefficient predict a linear relation between $1/S$ and concentration. Accordingly the data of Fig. 3 have been plotted in this manner in Fig. 4. It is apparent that the linear relationship does not hold at high concentrations; but at low concentrations extrapolation, assuming the linear relation to hold, yields the value for S°_{20} of 182 S.

If we make two assumptions, namely, that the molecular weights of DNA in ethanol and in 0.2 *M* NaCl are identical and that a linear extrapolation of the low concentration part of the curve shown in Fig. 4 is valid, we may calculate the approximate ratio of the frictional coefficients of DNA in the two solvents. The first assumption rests on the work of GEIDUSCHEK AND GRAY[5] discussed earlier. The second assumption appears from the results to be justifiable, but if it is in error, it is apparent from the general shape of the curve that the extrapolation value of S_{20} will most probably be an under-estimate of the true value.

From a study of DNA in 0.2 *M* NaCl, DOTY[1] gives values for S°_{20} and $\bar{V}$ of 22.5 S and 0.55 respectively. The value of the frictional coefficient, f, in terms of the molecular weight M of the DNA molecule is then given by

$$f_{0.2\,M\ \mathrm{NaCl}} = \frac{\mathrm{M}\,(1 - \bar{V}\rho)}{S^0_{20}} = \frac{\mathrm{M} \times 0.45}{22.5}$$

Similarly for DNA in ethanol using our approximate extrapolated value for S°_{20} of 182 S and the value of 0.38 for the partial specific volume (which was determined on the solutions used in the sedimentation experiments), we obtain, assuming an identical value for the molecular weight

$$f_{\mathrm{ethanol}} = \frac{\mathrm{M}\,(1 - \bar{V}\rho)}{S^0_{20}} = \frac{\mathrm{M} \times 0.69}{182}$$

which gives

$$\frac{f_{\mathrm{ethanol}}}{f_{0.2\,M\ \mathrm{NaCl}}} = 0.19$$

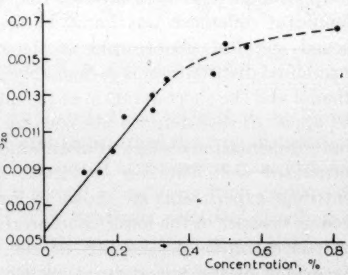

Fig. 4. The concentration dependence of the reciprocal of the sedimentation coefficient of DNA in 95% ethanol.

It is thus apparent that the molar frictional coefficient of DNA in ethanol has a value approximately one-fifth of that in 0.2 *M* NaCl.

The theories of BRINKMAN[14], DEBYE AND BUECHE[15] and KIRKWOOD AND RISEMAN[16], which attempt to describe the hydrodynamic behaviour of flexible chain macromolecules, all give rise to equations for the frictional coefficient that may be approximated by the expression

$$f = A\mathrm{M}^{\varepsilon} \qquad 1/2 \leqslant \varepsilon \leqslant 1$$

where the values of the "adjustable constants" A and ε depend on how much the flow of solvent through the polymer coil is restricted. For an open, very free-draining coil, ε approaches 1, whilst for a collapsed, non free-draining coil, ε approaches ½. It can thus be seen that if our assumptions are correct, and in particular if the molecular weight is constant in the two different media, the smaller value of f for an ethanolic solution must correspond to a decrease in the exponent; *i.e.*, the configuration of the molecular coil has changed from open free-draining to collapsed non free-draining. This conclusion is in agreement with the conclusions of Geiduschek and Gray. A consequence of the compact configuration of the DNA molecule in ethanol should be that, compared with aqueous solutions, if the DNA is polydisperse, a wider distribution of sedimentation coefficients will be observed.

It can be seen in Fig. 1 that the sedimentation boundaries observed for ethanolic DNA solutions spread rapidly, so it is of interest to examine the distribution of sedimentation coefficients, preferably at infinite dilution. Four graphs of refractive index gradient against distance from the centre of rotation of the cell were obtained at regular intervals during each sedimentation experiment. These graphs were then converted into apparent normalised sedimentation coefficient distributions $[g^*(S)]$ by the method of Williams, Baldwin, Saunders and Squire[17]. A single apparent normalised distribution showing the apparent distribution of the sedimentation coefficients of the sedimenting species on a refractive index concentration scale was obtained from each photograph; the effects of the radial increase in centrifugal field and dilution due to the sector shape of the cell having been eliminated. The only difference between these apparent distributions obtained at different times in a given centrifuge run is the time for which diffusion has been taking place across the initial sharp boundary. It is of interest that in the case of DNA dissolved in ethanol no significant difference was found between apparent distributions derived from the second and last photographs of the centrifuge run. The similarity between the normalised distributions is presumably due to the low diffusion coefficient of DNA in ethanol and the short duration of the ultracentrifuge runs. Consequently the mean of the apparent distributions obtained at a given concentration may be taken as the true sedimentation coefficient distribution $[g(S)]$ free from the effects of diffusion. The normalised sedimentation coefficient distributions obtained from typical ultracentrifuge experiments are shown in Figs. 5–7. It can be seen that the distributions become broader in the lower concentration experiments, this being a consequence of the concentration dependence of the sedimentation coefficient which leads to a sharpening of the boundary at higher concentrations. The most desirable way of presenting the results of such a boundary spreading analysis is in terms of $g(S^\circ)$, the distribution of sedimentation coefficients extrapolated to infinite dilution and thus

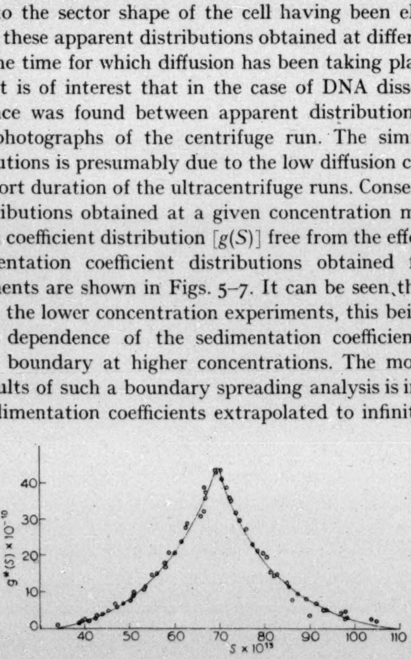

Fig. 5. Apparent normalised sedimentation coefficient distributions of DNA in 95% ethanol. Concentration 0.47%. Points derived from all five sedimentation photographs.

free from the effects of concentration dependence. This can only be done satisfactorily in a system where the dependence of S on concentration is linear, either by direct extrapolation or by application of the theory of BALDWIN[18]. The decidedly non-linear behaviour described here for DNA in ethanol precludes such a course in this case;

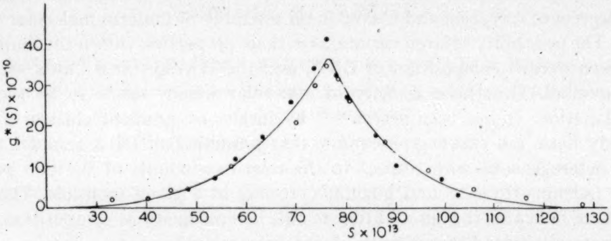

Fig. 6. Apparent normalised sedimentation coefficient distributions of DNA in 95 % ethanol. Concentration 0.30 %. ○, second photograph; ●, fifth photograph.

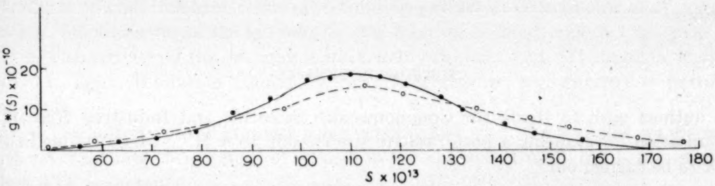

Fig. 7. Apparent normalised sedimentation coefficient distributions of DNA in 95 % ethanol. Concentration 0.12 %. ○, second photograph; ●, fifth photograph.

the best that can be achieved is to consider the distribution obtained at the lowest concentration used, whilst acknowledging the probability that the true distribution is rather wider. On examination of Figs 5–7 it is seen that at the lowest concentration at which experiments were made there was a spread of sedimentation coefficients from 50 to 170 S with a maximum at 110 S. This contrasts with the distribution obtained by SCHUMAKER AND SCHACHMAN[19] for DNA in 0.2 M aqueous NaCl at a concentration of 0.001 %, where they found a distribution of sedimentation coefficients from 10 to 60 S of asymmetric shape with a maximum at about 20 S. However, the two distributions are not strictly comparable since the distribution in aqueous solution is effectively at zero concentration whereas the distribution in ethanol is not. Bearing in mind the much higher concentration of the distribution for DNA in ethanol it is probable that the distribution at zero concentration would be rather wider than the distribution for aqueous solutions, in terms of the ratio of the S values of the fastest to the slowest sedimenting species. In spite of this incompatibility it is possible to assert that calf thymus DNA shows a broad distribution of sedimentation coefficients which, in view of the collapsed state of the molecule compared with aqueous solutions, reflects heterogeneity of molecular weight in confirmation of the results of SCHUMAKER AND SCHACHMAN[19].

Two other possible reasons for the wide spread of sedimentation coefficients in ethanolic solution may be advanced. In ethanolic solution, random aggregates of

DNA molecules might be formed; in this connection is it significant that GEIDUSCHEK AND GRAY[5] determined the molecular weight of DNA in ethanol by light scattering, and found it to be within 15 % of the value obtained in dilute saline solution. As light scattering yields a weight-average molecular weight, the presence of aggregates seems unlikely. It might be that the sedimentation coefficient distribution represents different degrees of solvation and coiling in an assembly of uniform molecular weight molecules. The possibility of large variations in these properties, within the limitations of the known overall composition of DNA, and the WATSON AND CRICK structure seem quite small. Thus heterogeneity of molecular weight seems to be the most likely explanation. It has been shown[20, 21] by means of gradient elution of DNA from weakly basic ion exchange columns, that a number of DNA samples are remarkably heterogeneous with respect to the relative amounts of the two possible base pairs (adenine-thymine and guanine-cytosine) in a given molecule. The work described here indicates that in addition to this heterogeneity of composition, there is almost certainly great heterogeneity of molecular weight.

It may thus be concluded that the ultracentrifugal analysis of DNA in ethanolic solution is a method that may be used to examine the heterogeneity of DNA preparations.

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DER PHYSIOLOGISCHE ERSCHLAFFUNGSFAKTOR UND DIE MUSKELGRANA

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SUMMARY

The physiological relaxing factor produced by the muscle granules

1. Practically the entire granule fraction which inhibits ATP-splitting and contraction is precipitated from crude rabbit-muscle extract by centrifuging at 25,000 × *g* for 1 h. The diameter of the granules in this fraction is about 1000 Å (700–3000 Å).

2. The activity of the granules is irreversibly destroyed by SH-reagents, thymol and u.v. light. Inhibition caused by certain detergents, *e.g.* antarox is partially reversible.

3. The activity of the granules is irreversibly lost when the granules are digested with several times their weight of trypsin in the absence of ATP, but is not affected when ATP is present.

4. Before they can produce inhibition, the granules must be activated by ATP and Mg in the absence of Ca. Activation takes only a few minutes, and occurs normally during the experiments themselves.

5. The inhibition of ATP splitting and contraction of fibrils or actomyosin floccules produced by the granules ceases as soon as the granules are removed.

6. Thus, the granules do not withdraw any functionally essential substances from the contractile proteins, nor do they release any stable contraction-inhibiting substance to the actomyosin or to the solution.

7. Over a wide concentration range, the effect of the granules does not depend on their absolute concentration, but on the granule/fibril ratio, in spite of the fact that the granules are not bound to the fibrils. Hence, the granules can not be identical with the inhibitor which reacts directly with the actin- and myosin-filaments.

8. It seems probable, however, that the granules release a labile inhibitor, since their relaxing effect can be exhausted by repeated washing with fibril suspensions. During such washings, the fibrils remove no granules, but merely take away their activity.

9. It is shown that all relaxing and inhibiting effects of the granules in actomyosin gels are brought about only by dissociation of the actomyosin in the presence of ATP and granules. The L-myosin-ATPase as well as the ability for and the state of polymerisation of the actin are not affected by the relaxing granules.

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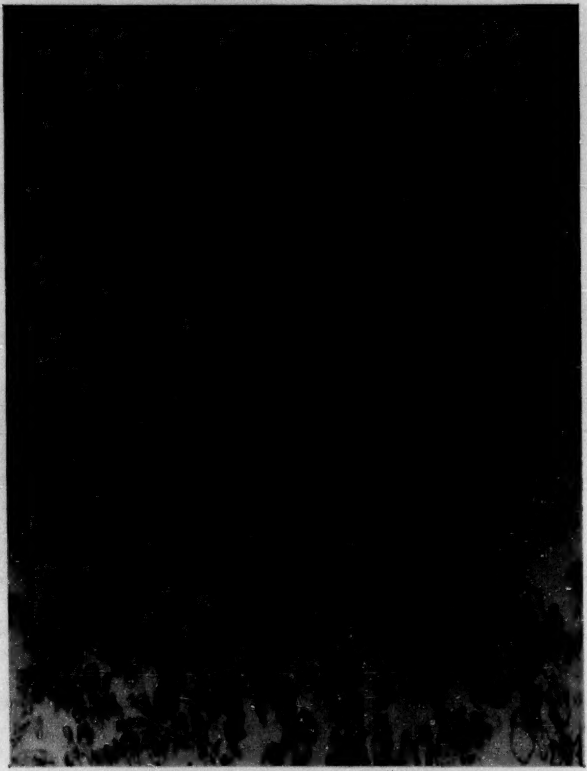

Fig. 1. Dünnschnitt durch einen Grananiederschlag. Die Grana wurden durch einstündiges Zentrifugieren bei 25,000 × *g* niedergeschlagen und in Osmiumsäure fixiert. Dünnschnitt und Aufnahme wurde von H. E. HUXLEY, London, gemacht.

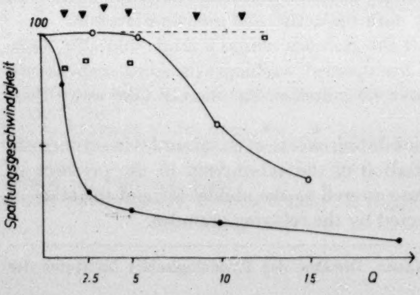

Fig. 2. Die Schädigung der Grana durch verschiedene Reagenzien und u.v. Licht. Ordinate: Geschwindigkeit der ATP-Spaltung durch Fibrillen in Prozenten der ungehemmten Spaltung. Abszisse: Grana-N · Fibrillen-N^{-1} · 100 = Q. ● Grana ohne Vorbehandlung. ○, 30 mg Grana mit 10 mg Digitonin 15 Min inkubiert; □, 10 mg Granaprotein mit 10 mg Antarox 15 Min inkubiert; ▼, 10 mg Granaprotein 20 Min mit u.v. Licht bestrahlt.

EINLEITUNG

Es ist seit einer Reihe von Jahren bekannt, dass kontrahierte Aktomyosinstrukturen erschlaffen, sobald die Spaltung des anwesenden ATP durch ATPase-Gifte unterdrückt wird, und dass die Strukturen im erschlafften Zustand bleiben, solange diese Hemmung andauert[1]. Erst seit dem Symposium über Molecular Biology[2] und dem Symposium der New York Academy[3] ist es bekannt, dass der von MARSH[4,5] entdeckte und von BENDALL[6,7] identifizierte natürliche Erschlaffungsfaktor nicht durch Blockierung der Myosin-ATPase, sondern durch Verwandlung der Aktomyosin-ATPase in die L-Myosin-ATPase wirkt. Diese Erkenntnis beruht experimentell auf den Ergebnissen der vorliegenden Arbeit.

Die Ansicht, dass nicht nur die Beeinflussung der ATP-Spaltung, sondern auch die Restitution des ATP durch alle ATP regenerierenden Systeme[8–10] Erschlaffung bewirkt, ist inzwischen durch PORTZEHL[11] sowie durch BRIGGS UND PORTZEHL[12] widerlegt. Darüber hinaus hat zuerst PORTZEHL[13] (vergl. WEBER[14]) gezeigt, dass das wirksame Prinzip des physiologischen Erschlaffungsfaktors eng mit gewissen Grana verbunden ist.

Ein Hinweis auf diese Tatsache ergibt sich aus der älteren Mitteilung von KUMAGAI *et al.*[15], die Meyerhof–Kielley-ATPase sei eine Komponente des Erschlaffungsfaktors. Das Ergebnis von PORTZEHL[13] wurde sehr schnell von BENDALL[16] bestätigt. Zu dem gleichen Ergebnis kamen kurze Zeit später unabhängig EBASHI[17] sowie LORAND[18,19].

In dieser Mitteilung wird weiterhin über Beobachtungen berichtet, die dafür sprechen, dass die Grana nicht mit dem Erschlaffungsfaktor identisch sind, sondern den Faktor abgeben und ihn wahrscheinlich vorher produzieren. Es werden Angaben gemacht über die Bedingungen der Abgabe (Produktion) dieses Faktors durch die Grana und seine Wirkung.

Die Natur der Grana

Wird Blendor behandelter Muskelbrei mit einer Lösung von 0.1 M KCl bei pH 7.0 (Phosphatpuffer, 0.005 M) in Anwesenheit von 0.005 M Oxalat extrahiert, so werden die wirksamen Grana zusammen mit anderen Grana ausgewaschen. Die wirksamen Grana gehören der Fraktion an, die durch Zentrifugieren mit 8000 × g (20 Min) noch nicht und mit 25,000 × g (60 Min) fast vollständig niedergeschlagen wird (vergl. LORAND *et al.*[18] und BRIGGS *et al.*[20]).

Aus Fig. 1 geht hervor, dass die Teilchen oder Bläschen dieser Fraktion einen mittleren Durchmesser von ~ 1000 Å haben (Fig. 1; 700–3000 Å).

Solche Grana bleiben in einer 50 % Glycerin–Wasser-Mischung mit einem Gehalt von 0.1 M KCl, 0.005 M Oxalat, pH 7.0, bei Temperaturen um Null Grad etwa 10 Tage aktiv. Die Wirkung erlischt sofort, wenn in der Granasuspension oberflächenaktive Stoffe oder SH-Reagenzien (Salyrgan* und N-Äthylmaleimid) in genügender Konzentration anwesend sind. Die entsprechenden Angaben von LORAND *et al.*[18] und GERGELY *et al.*[21] für Thymol und Desoxycholsäure werden bestätigt und um Antarox** und Digitonin als weitere oberflächenaktive Granagifte vermehrt (Fig. 2). Es ist für Thymol und Salyrgan (Tabelle I) geprüft, dass die Granaschädigung irreversibel ist. Für Antarox ist die Schädigung partiell reversibel. Auch die Bestrahlung mit u.v.-Licht in genügender Intensität vernichtet die Granawirkung irreversibel.

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* Salyrgan = Mersalyl, Salicyl-(α-Hydroxymercuri-β-Methoxypropyl)-amidoacetat.

** Nonyl–Phenoxy–Polyoxyäthylen–Äthanol.

TABELLE I

DIE IRREVERSIBLE VERGIFTUNG DER GRANA DURCH SALYRGAN

Vorbehandlung der Grana mit Salyrgan	*Nach Entgiftung der Grana durch Cystein* *ATP-Spaltung durch Fibrillen in Prozenten der ungehemmten Spaltung*	 *Spaltungshemmung in Prozenten der maximalen Hemmung*
0	16	100
$3 \cdot 10^{-6}$	42	69
10^{-5}	70	36
$3 \cdot 10^{-5}$	76	28
10^{-4}	90	12
$3 \cdot 10^{-4}$	100	0

Auch eine 10 Min-Inkubation der Grana mit calciumarmem Trypsin macht die Grana unfähig, die Spaltung von 0.005 *M* ATP durch Fibrillen zu hemmen. Findet dagegen die Verdauung der Grana in Gegenwart von 0,005 *M* ATP statt, so ist die spaltungshemmende Aktivität nach 40 Min noch voll erhalten (Fig. 3).

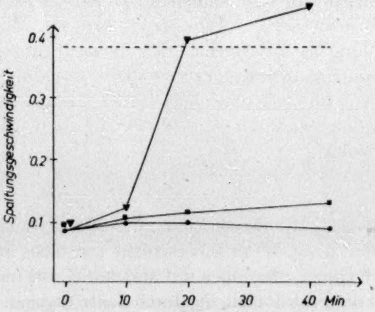

Fig. 3. ATP schützt die Granawirkung gegen Trypsinverdauung. Ordinate: Geschwindigkeit der ATP-Spaltung durch Fibrillen μMol P/mg Ew.·Min. Abszisse: Dauer der Vorbehandlung der Grana. ●, Grana in Mg–ATPat-haltiger Lösung inkubiert; ▼, Grana in ATP-freier Lösung mit Trypsin behandelt; ■, Grana in Gegenwart von $5 \cdot 10^{-3}$ *M* ATP mit Trypsin behandelt. – – –, Fibrillenspaltung ohne Grana. Grana N·Fibrillen $N^{-1} \cdot 100 = 5.3$; 0.2 mg Grana wurden mit 1 mg Trypsin behandelt bei pH 7.0; Temperatur, 22°.

Grana und Rohextrakt

Wird die hemmende Wirkung der Grana und des Rohextraktes auf die ATP-Spaltung und ATP-Kontraktion von gewaschenen Muskelfibrillen vergleichend gemessen, so ergeben sich folgende Wirkungen und Wirkungsbedingungen, sowohl für die Granasuspension wie für den Rohextrakt: (a) Die Hemmung der ATP-Spaltung und ATP-Kontraktion durch Grana und Rohextrakt ist umso höher je höher die ATP-Konzentration ist[22]. Die Hemmung nimmt auch in Gegenwart hoher Granakonzentrationen mehr und mehr ab, wenn die ATP-Konzentration entsprechend verkleinert wird (Fig. 4). (b) Die hemmende Wirkung verschwindet ferner, sobald Spuren von Calciumionen anwesend sind[5]. Die notwendige Calciumkonzentration wächst mit zunehmender Konzentration der Grana (Fig. 5). (c) Grana und Rohextrakt wirken nur spaltungshemmend und erschlaffend, falls Magnesiumionen anwesend sind. Die Spaltungshemmung erreicht ihren Maximalwert, wenn die Magnesiumkonzentration 10^{-3} *M* ist (Fig. 6). (d) Wie schon PORTZEHL[13] festgestellt hat, ist die Hemmwirkung verschiedener Verdünnungen eines Rohextraktes und der aus diesem Rohextrakt isolierten Grana auch quantitativ gleich oder fast gleich.

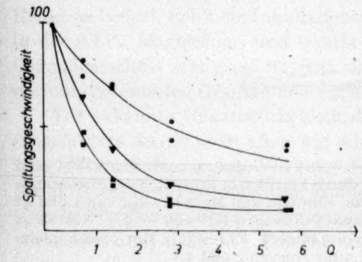

Fig. 4. Die Abhängigkeit der Granawirkung von der ATP-Konzentration. Ordinate: Geschwindigkeit der ATP-Spaltung durch Fibrillen in Prozenten der ungehemmten Spaltung. Abszisse: Grana-N · Fibrillen-N^{-1} · 100 = Q; [ATP], ●, $1 \cdot 10^{-3}$ *M*; ▼, $2.5 \cdot 10^{-3}$ *M*; ■, $5 \cdot 10^{-3}$ *M*. [Mg^{++}], $2.5 \cdot 10^{-3}$ *M*.

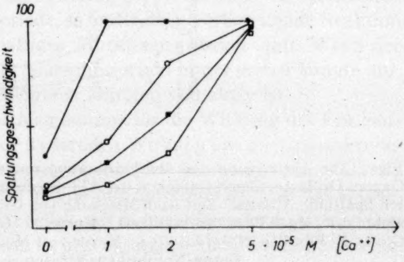

Fig. 5. Die Inaktivierung der Granawirkung durch Calcium. Ordinate: Geschwindigkeit der ATP-Spaltung durch Fibrillen in Prozenten der ungehemmten Spaltung. Abszisse: Gesamtcalcium. Die Konzentration des freien Calciums ergibt sich aus der Anwesenheit von $5 \cdot 10^{-3}$ *M* ATP und $5 \cdot 10^{-3}$ *M* Oxalat. Grana-N · Fibrillen-N^{-1}. 100: ●, 2, ○, 4; ■, 6; □, 8.

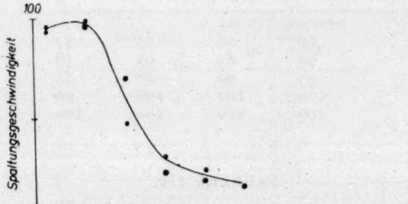

Fig. 6. Der Einfluss der Magnesiumkonzentration auf die Granawirkung. Ordinate: Geschwindigkeit der ATP-Spaltung durch Fibrillen in Gegenwart von Grana (Grana-N · Fibrillen-N^{-1} · 100 = 2.5) in Prozenten der Spaltungsgeschwindigkeit ohne Grana. Abszisse: Magnesiumkonzentration.

Die Bedingungen der Granaaktivierung

Wird eine so kleine Menge von Grana* mit Fibrillen gemischt, dass in einem Magnesium-, ATP- und Oxalat-haltigen System eine unvollständige oder eben vollständige Hemmung der ATP-Spaltung eintritt, so setzt die Hemmung nicht schlagartig

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[*] Die Angaben dieses Abschnittes gelten für Grana, die mehr als 2 Tage nach dem Tod des Tieres verwendet werden. Ganz frische Grana verhalten sich schon ohne Inkubation so wie 2 oder mehr Tage alte Grana nach Inkubation.

ein, sondern steigt über eine Dauer von 5 bis 10 Min an (Fig. 7, Kurve 1). In dem Masze, in dem die zugesetzte Granamenge vergröszert wird, verkürzt sich die Zeit, in der der stationäre Wert der Hemmung erreicht wird (Fig. 7, Kurve 4). Ebenso setzt die Granawirkung sehr schnell ein, wenn die Grana vor ihrer Mischung mit den

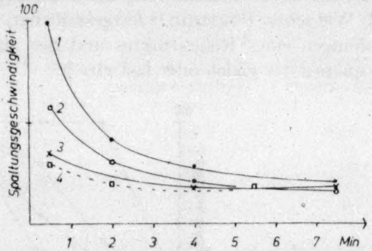

Fig. 7. Die Aktivierung und die Aktivierungszeit der Grana in Gegenwart von Mg-ATPat und Oxalat. Ordinate: Geschwindigkeit der ATP-Spaltung durch Fibrillen in Prozenten der ungehemmten Spaltung. Abszisse: Zeit nach Mischung von Grana, Fibrillen und Mg-ATP. Kurven 1 und 4: nicht mit Mg–ATPat vorinkubiert. Kurve 1: Grana-N·Fibrillen-N^{-1}·100 = 1.5. Kurve 4: Grana N·Fibrillen N^{-1}·100 = 5.0. Kurve 2: 1 Min vorinkubiert. Kurve 3: 2 Min vorinkubiert. Grana-N·Fibrillen-N^{-1}·100 = 1.5 für Kurven 2 und 3.

TABELLE II

DIE WIRKUNG CALCIUM-BINDENDER SUBSTANZEN AUF DIE AKTIVIERUNG DER GRANA

In der	*Hemmung der Fibrillen-ATPase durch Grana in Prozenten der maximalen Hemmung nach 3 Min Vorbehandlung mit Mg-ATPat und*				*Ohne Vorbehandlung*
	Citrat ($5 \cdot 10^{-3}$ M)	*Oxalat ($5 \cdot 10^{-3}$ M)*	*EDTA ($5 \cdot 10^{-3}$ M)*	—	
1. Min	66	68	75	57	34
2. Min	90	85	93	76	60
3. Min	95	90	98	85	78
4. Min	100	100	100	90	87
8. Min	100	100	100	100	100

TABELLE III

BEDARF DER GRANAAKTIVIERUNG AN MAGNESIUM, ATP UND OXALAT

Die 3-Min inkubierten Grana werden in einem kleinen Volumen gleichzeitig mit den Fibrillen einem normalen Spaltungsansatz (ATP = Mg^{++} = Oxalat = $5 \cdot 10^{-3}$ *M*) zugesetzt. Die Spaltungszeit beträgt 3 Min. Grana-N·Fibrillen-N^{-1}·100 = 1.6–4.0.

Konzentration der variierten Substanz im Inkubations-Ansatz M*	*Spaltung in Prozenten der ungehemmten Spaltung bei Variation* von*		
	ATP	*Magnesium*	*Oxalat*
0	90	85	67
10^{-4}	85	63	65
$3 \cdot 10^{-4}$	70	50	65
10^{-3}	60	42	52
$5 \cdot 10^{-3}$	30	38	42

* Die beiden nicht variierten Komponenten sind in optimalen Konzentrationen vorhanden.

Fibrillen für mindestens 3 Min in einer ATP- und Magnesium-haltigen Lösung inkubiert werden, deren Calciumspuren durch Oxalat, Citrat oder EDTA weitgehend gebunden sind (Fig. 7, Kurven 2 und 3; Tabelle II). Der Erfolg der Inkubation verschwindet, wenn eine der notwendigen Komponenten in der Inkubationslösung fehlt. Die Intensität der Aktivierung der Grana während der Inkubationszeit wächst mit der Konzentration aller drei Komponenten, d.h. des ATP, des Magnesiums und des Oxalats bis zu den höchsten untersuchten Konzentrationen von $5 \cdot 10^{-3}$ *M* an (Tabelle III).

Die Inkubationseffekte zeigen, dass die Wirkung der Grana einer vorbereitenden Reaktion bedarf. Fehlt die Inkubationsperiode, so findet diese vorbereitende Reaktion in der ATP-, Magnesium- und Oxalat-haltigen Fibrillensuspension statt. Wenn der Granaüberschuss sehr grosz ist, tritt volle Hemmung schon in der ersten Minute auf, obwohl das einzelne Granum erst nach mehreren Minuten voll aktiv ist.

Die bekannte Unentbehrlichkeit des Magnesiums für die Wirkung des Erschlaffungsfaktors beruht nicht allein auf seiner fördernden Wirkung auf die Granaaktivierung. Denn die hemmende Wirkung der Grana bleibt auch dann aus, wenn während der aktivierenden Vorinkubation genügend Magnesium anwesend ist, aber das Magnesium in Fibrillen-haltigen Spaltungsansatz selbst nur in ungenügender Konzentration vorhanden ist (Tabelle IV). Das Magnesium ist also zusätzlich auch für die hemmende Wirkung der bereits aktivierten Grana notwendig.

TABELLE IV

WIRKUNGSLOSIGKEIT MIT Mg-ATP VORBEHANDELTER GRANA IN MAGNESIUM-ARMEN SPALTUNGSANSÄTZEN

$Mg^{++} = 2 \cdot 10^{-5}$ *M*, Grana-N/Fibrillen-N · 100 = 2.5.

*ATP-Spaltung**	
Ohne Grana	*Mg-ATP-inkubierten Grana Mit 2 · 10⁻⁵ M*
0.28	0.28
0.30	0.30
0.18	0.19
0.175	0.16

* μMol P/mg Fibrillen Protein/Min.

Das Ende der Granawirkung

Man kann die Grana aus einer ATP- und Magnesium-haltigen Mischung mit Fibrillen entfernen. Die Fibrillen werden abzentrifugiert; aus dem granahaltigen Überstand werden die Grana mit der Ultrazentrifuge beseitigt; die abzentrifugierten Fibrillen werden in dem granafreien Überstand resuspendiert. Es zeigt sich dann, dass die Hemmung der ATP-Spaltung durch die Fibrillen nach der Entfernung der Grana aufgehoben ist. Im granafreien Überstand spalten die Fibrillen wieder ungehemmt und kontrahieren sich gleichzeitig. Das gleiche Resultat ergibt sich, wenn die aus der Granasuspension abzentrifugierten Fibrillen in eine frisch bereitete ATP-Lösung gebracht werden (Tabelle V).

TABELLE V

ENTHEMMUNG DER FIBRILLEN DURCH DIE ENTFERNUNG DER GRANA

ATP-Spaltung durch Fibrillen in Prozenten der ungehemmten Spaltung			
Vor Entfernung der Grana	*Nach Entfernung der Grana und Resuspension der Fibrillen in frischer ATP-Losung*	*Vor Entfernung der Grana*	*Nach Entfernung der Grana und Resuspension der Fibrillen in der Ausgangslösung*
19	103	12	106
20	99	19	89
15	94	20	106
17	92	23	94
		17	100
		12	100

Dieser einfache Versuch schliesst eine ganze Reihe von Mechanismen der Granawirkung aus.

Er zeigt, dass die Grana nicht dadurch hemmend wirken können, dass sie aus den Fibrillen eine Substanz entfernen, die für die Magnesium-aktivierte ATP-Spaltung und die Kontraktion notwendig ist. Denn dann sollte die Kontraktion der Fibrillen und die ATP-Spaltung auch nach Entfernung der Grana gehemmt bleiben, weil zusammen mit den Grana auch dieser kontraktions- und spaltungswichtige Stoff entfernt ist. Die Wirkung der Grana beruht also auch nicht auf der Entfernung von Calcium oder Magnesium, wie gelegentlich angenommen wurde[23]*.

TABELLE VI

*Der Einflusz der Grana auf den Calciumgehalt der Myofibrillen**	
Vor Inkubation mit Grana	*Nach Inkubation mit Grana*
12.6	11.6
12.7	11.8
12.8	11.9
11.6	11.3

* μMol Ca^{++}/Gr. Prot.

Das Ende der Granawirkung mit der Entfernung der Grana schliesst aber auch aus, dass die Grana an die Fibrillen stabile Hemmstoffe abgeben. Auch in diesem Falle müsste die Granawirkung die Granaentfernung überdauern. Selbst wenn mehrere Stoffe bei der Hemmung zusammenwirken sollten, treffen alle diese Stoffe wieder zusammen, sobald die Fibrillen in der gleichen, nur von den Grana befreiten Lösung resuspendiert werden, in der sie sich während der Hemmungsperiode befunden haben. Gegen eine Abgabe von Hemmstoff ausschliesslich an die Lösung spricht ferner die Beobachtung, dass der granafreie Überstand ATP-Magnesiumhaltiger Granasuspen-

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[*] Auch die Bestimmung des Calciumgehaltes von Fibrillen vor und nach Inkubation mit Grana, Magnesium und ATP zeigt, dass die Grana den Fibrillen Calcium nicht oder kaum entziehen (Tabelle VI), obwohl die Fibrillenkontraktion und die ATP-Spaltung in den granahaltigen Ansätzen gehemmt sind.

TABELLE VII

WIRKUNGSLOSIGKEIT DER GRANAFREIEN ÜBERSTÄNDE VON Mg–ATPat-HALTIGEN GRANASUSPENSIONEN

ATP-Spaltung durch unbehandelte Fibrillen in Prozenten der ungehemmten Spaltung in Gegenwart von	
Granafreien Lösungen, abgetrennt aus Mg-ATPat enthaltenden Granasuspensionen	*Granafreien Lösungen, abgetrennt aus Mg-ATPat + Fibrillen haltigen Granasuspensionen*
97	105
102	103
95	96

sionen nicht mehr wirkt. Der Überstand ist auch dann wirkungslos, wenn die Granasuspension zusätzlich mit Fibrillen inkubiert war (Tabelle VII).

Infolgedessen scheint nur folgende Alternative übrig zu bleiben. Entweder: die Grana reagieren wie eine chemische Substanz mit den Aktin- und Myosinfilamenten, deren enzymatische Interaktion (als Aktomyosin-ATPase) und deren mechanische Interaktion (als Kontraktion) sie verhindern. Oder: die Grana geben eine hemmende Substanz ab, die so labil ist, dass ihr Spiegel sehr schnell auf Null sinkt, falls die Substanz von den Grana nicht mehr nachgeliefert wird.

Es ist unwahrscheinlich, dass die Grana mit der Erschlaffungssubstanz identisch sind. Denn von einer Erschlaffungssubstanz müssen auch die innersten Filamente der Fibrillen erreicht werden. Grana mit einem Durchmesser von 1000 Å aber können nicht in das Innere der Fibrillen eindiffundieren.

Ferner müsste die Hemmungswirkung der Grana nach dem Massenwirkungsgesetz von der Granakonzentration abhängen, wenn die Grana selbst als Hemmstoff mit den Fibrillen reagierten. Die Intensität der Hemmung hängt aber nur dann von der Granakonzentration ab, wenn die Granakonzentration in den Ansätzen sehr klein ist ($\gtrsim$ 0.003 mg Granaprotein/ml). Ist die Granakonzentration gröszer, so hängt

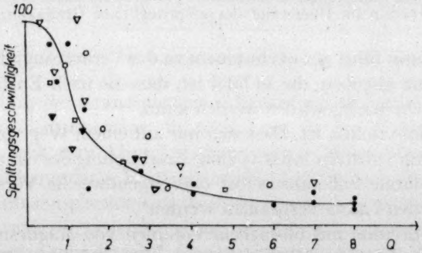

Fig. 8. Die Abhängigkeit der Fibrillen–ATPase von der Proportion der Grana zu den Fibrillen. Ordinate: Geschwindigkeit der ATP-Spaltung durch Fibrillen, ungehemmte Spaltung = 100. Abszisse: Grana-N·Fibrillen-N^{-1}·100 = Q. ○, 0.26 mg Fib.Prot./ml; ●, 0.38 mg Fib.Prot/ml; □, 0.8 mg Fib.Prot/ml; ▼, 2.2 mg Fib. Prot/ml; ▽, 3.8 mg Fib.Prot/ml. Die zu den angegebenen Fibrillenkonzentrationen gehörige Granakonzentration beträgt 0.0025 mg/ml für die niedrigen Q-Werte und die niedrigsten Fibrillenkonzentrationen. Sie wächst mit steigenden Q-Werten und steigenden Fibrillenkonzentrationen bis 0.25 mg/ml.

die Intensität der Hemmung von der Proportion der Grana zu den Fibrillen ab (Q-Wert = Grana-N · Fibrillen-N^{-1} · 100). Dies zeigt Fig. 8, in der bei konstantem Q-Wert die absolute Konzentration der beiden Komponenten bis zum zehnfachen variiert wird, ohne dass sich die ATP-Spaltung für den gegebenen Q-Wert deutlich dabei ändert. Dieser Befund ist mit der Gültigkeit des Massenwirkungsgesetzes für die Reaktion zwischen Grana und Fibrillen nur unter der Voraussetzung vereinbar, dass die Fibrillen die Grana mehr oder weniger unabhängig von ihrer absoluten Konzentration vollständig oder fast vollständig binden.

Das aber ist nicht der Fall. Denn die freien Grana lassen sich im Überstand nachweisen. Der Überstand hemmt nach dem Abzentrifugieren der Fibrillen frische Fibrillen in genau dem gleichen Umfang, in dem die ursprünglichen Fibrillen vor dem Abzentrifugieren gehemmt wurden, wenn die Grana-Fibrillen-Proportion ≥ 1.5 ist (Fig. 9). Mit kleineren Q-Werten als 1.5 ist eine hemmende Wirkung weder vor noch nach dem Abzentrifugieren der Fibrillen deutlich nachzuweisen. Der Überstand enthält also nach dem Abzentrifugieren der Fibrillen praktisch ebensoviel Grana wie vorher.

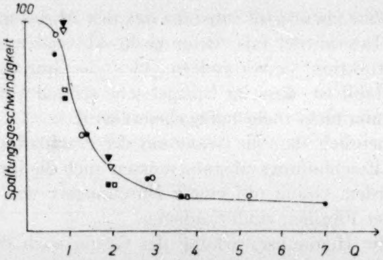

Fig. 9. Die Granawirkung im Überstand einer Grana–Fibrillen-Mischung nach Abzentrifugieren der Fibrillen. Abszisse: Q-Werte. Ordinate: Geschwindigkeit der ATP-Spaltung durch Fibrillen, ungehemmte Spaltung = 100. Offene Symbole, in Anwesenheit von Grana aus dem Überstand; gefüllte Symbole, in der ursprünglichen Grana–Fibrillen-Mischung. Die Grana–Fibrillen-Proportion bezieht sich auf die ursprüngliche Mischung vor dem Abzentrifugieren der Fibrillen. Der Q-Wert der Mischung des Überstandes mit frischen Fibrillen ist unter der Annahme berechnet, dass die Granakonzentration im Überstand der ursprünglichen Granakonzentration gleich ist.

Diese Beobachtung führt per exclusionem zu der Vermutung, dass die Grana eine Erschlaffungssubstanz abgeben, die so labil ist, dass sie nach Entfernung der Grana sehr schnell nicht mehr nachgewiesen werden kann.

Ob diese Annahme richtig ist, lässt sich nur auf einem Wege prüfen. Eine intensive Abgabe der labilen Substanz muss zu einer Erschöpfung der Granawirkung führen. Denn entweder die labile Substanz selbst oder irgendwelche Vorstufen der labilen Substanz müssen in den Grana verbraucht werden*.

Werden Grana dreimal mit 60-fachem Volumen von Magnesium-haltiger ATP-Lösung gewaschen, so ist keine Wirkungsabnahme nachweisbar (Tabelle VIII). Dagegen lässt sich die Wirkung der Granalösung sofort weitgehend erschöpfen, wenn wenige Grana mit sehr viel Fibrillen gewaschen werden.

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* Eine Erschöpfung der Grana braucht trotz Abgabe einer labilen Substanz nicht einzutreten, wenn das immer gegenwärtige ATP die Vorstufe der Erschlaffungssubstanz ist oder wenn die Grana die zerstörte Erschlaffungssubstanz immer wieder restituieren.

TABELLE VIII

GLEICHHEIT DER WIRKUNG UNGEWASCHENER UND SEHR GRÜNDLICH MIT OXALAT SOWIE Mg–ATP-OXALAT GEWASCHENER GRANA

Grana N/Fibrillen N · 100	*ATP-Spaltung in µMol P/mg Fibrillen Prot. · Min* Ungewaschene Grana	 Mit Oxalat gewaschene Grana *	 Mit Mg-ATP und Oxalat gewaschene Grana *
1	0.43	0.40	0.41
3	0.26	0.23	0.22
5	0.12	0.13	0.13

* Dreimal mit dem 60-fachen Volumen.

Dann genügt bisweilen schon eine einmalige Waschung für eine erkennbare und immer eine zweimalige Waschung für eine grosse Abnahme der hemmenden Granawirkung (Tabelle IX), obwohl bei der Prüfung der restierenden Granawirkung viele Grana mit wenigen Fibrillen gemischt werden (Q = 5, s. Tabelle IX).

TABELLE IX

ABNAHME DER GRANAWIRKUNG NACH WASCHUNG EINER KLEINEN GRANAMENGE MIT EINER GROSZEN FIBRILLENMENGE (Q = 0.75) IN Mg-ATPat-LÖSUNG

Anzahl der Waschungen	*Hemmung der ATP-Spaltung in Prozenten der maximalen Hemmung durch gewaschene Grana (gemessen mit Q = 5)* Gewaschen mit Fibrillen	 Gewaschen ohne Fibrillen
1	90	100
1	76	100
2	4	—
1	87	98
2	1	67
1	100	100
2	12	97
1	94	97
2	64	94

Diese Erschöpfung der Granasuspension beruht mit Sicherheit nicht darauf, das die sehr dichte Fibrillensuspension die Grana mit in den Niederschlag reisst. Der Überstand enthält nach dem Abzentrifugieren der Fibrillen ebensoviele Grana wie vorher, wie die nephelometrische Kontrolle ergibt (Tabelle X). Infolgedessen kann eine Erschöpfung der Granasuspension nur dann durch eine Verarmung an Grana erklärt werden, wenn man zusätzlich zwei Annahmen macht. (a) Nur eine sehr kleine Fraktion der suspendierten Grana gibt die labile Erschlaffungssubstanz ab. (b) Diese unmessbar kleine Fraktion wird von den Fibrillen selektiv in den Niederschlag mitgenommen.

Die zweite Annahme ist nicht sehr wahrscheinlich. Vor allem aber spricht das Experiment gegen die zweite Annahme.

Wenn wenige Grana mit sehr viel Fibrillen (Q = 0.5) in Abwesenheit von Magnesium-ATPat ein- bis zweimal gewaschen werden, so können die Grana während der

TABELLE X

KONSTANZ DER GRANAKONZENTRATION TROTZ WASCHUNG EINER KLEINEN GRANAMENGE MIT EINER GROSZEN FIBRILLENMENGE IN GEGENWART VON Mg-ATPat

Grana-Konzentration mg Prot./ml	*Trübung der Granalösung**	
	Vor Waschung mit Fibrillen	*Nach Waschung mit Fibrillen***
0.01	32	—
0.02	48	53
0.05	79	86
0.075	92	99
0.1	100	108

* Die Trübung der 0.01 % Granalösung ist gleich 100 gesetzt.

** Die Trübung ist korrigiert um den kleinen Trübungsbeitrag, der selbst von gut gewaschenen Fibrillen erzeugt wird. Er ist in jedem Versuch gesondert bestimmt. 0.1 mg Granaprotein/ml entspricht einem Q von 1.0.

Waschungen keine Erschlaffungssubstanz abgeben, weil die Grana nur in Gegenwart von Magnesium-ATPat aktiv sind (vergl. *Die Bedingungen der Granaaktivierung*). Tabelle XI zeigt, dass der granahaltige Überstand nach dem Abzentrifugieren der Fibrillen unverändert volle Hemmung hervorruft, wenn die Grana unter Normalbedingungen ($Q = 1.6$–3.0) mit Fibrillen und Magnesium-ATPat gemischt werden. Im inaktiven Zustand werden die Grana durch die Waschung mit einem Fibrillenüberschuss weder erschöpft noch aus dem Überstand entfernt.

TABELLE XI

KONSTANZ DER GRANAWIRKUNG NACH WASCHUNG EINER KLEINEN GRANAMENGE MIT EINER GROSZEN FIBRILLENMENGE IN ABWESENHEIT VON Mg-ATPat

Anzahl der Waschungen	*Grana-Fibrillen Proportion Q*	*Hemmung der ATP-Spaltung in Prozenten der maximalen Hemmung**	
		Nach Waschung mit Fibrillen	*Vor Waschung mit Fibrillen*
1	0.55	84	82
1	0.55	92	92
1	0.65	85	90
2	0.61 u. 0.5	92	82
2	0.61 u. 0.5	95	100

* Gemessen bei einer Grana-Fibrillen-Proportion Q, 1.6 bis 3.0.

Wird infolgedessen angenommen, dass die Erschöpfung der Granawirkung auf der Abgabe und Zerstörung eines labilen Inhibitors beruht, so ergibt sich die weitere Folgerung: Der labile Inhibitor wird nur dann in messbarem Umfang abgegeben, wenn er an Fibrillen gebunden wird. Denn sonst müsste auch eine Waschung mit reiner Magnesium-ATPat-Lösung zur Erschöpfung der Grana führen.

Wenn der Hauptteil des abgegebenen Inhibitors an die Fibrillen gebunden wird und wenn der Inhibitor labil ist, ist zu erwarten, dass die Hemmungswirkung nur von der Proportion der Grana zu den Fibrillen und nicht von der absoluten Konzentration noch von der Einwirkungszeit der Grana abhängt. (a) Die Labilität des

Inhibitors verhindert seine Akkumulation und damit ein Anwachsen der Hemmungswirkung mit der Einwirkungszeit der Grana. (b) Die Bindung eines "labilen" Inhibitors an die Fibrillen macht die Geschwindigkeit der Nachlieferung zum limitierenden Faktor der gebundenen Inhibitormenge, solange die Fibrillen nicht mit dem Inhibitor gesättigt sind. Die Menge des in der Zeiteinheit nachgelieferten Inhibitors steigt proportional mit der Menge der anwesenden Grana, während der für einen bestimmten Hemmungsgrad notwendige Bedarf proportional mit der Menge der Fibrillen ansteigt. Die absolute Konzentration von Grana und Fibrillen fällt aus dieser Betrachtung heraus; die Hemmung ist allein durch die Proportion Grana: Fibrillen bestimmt, solange nicht angenommen wird, dass ein wesentlicher Teil des Inhibitors frei in der Lösung zerstört wird (vergl. dazu Fig. 8).

Die Q-Abhängigkeit der Hemmung ist mit der Annahme einer labilen Erschlaffungssubstanz vereinbar, weil die Fibrillen die Erschlaffungssubstanz weitgehend oder fast vollständig zu binden scheinen. Die Q-Abhängigkeit der Hemmung spricht dagegen gegen eine unmittelbare Reaktion der Grana mit den Fibrillen, weil sich nachweisen lässt, dass die Fibrillen die Grana nicht wesentlich binden.

Der Erschlaffungsfaktor als Interaktions-Inhibitor

Es ist lange bekannt, dass der Erschlaffungsfaktor und das ATP zusammenwirken[22]. 1952 haben HASSELBACH UND WEBER erstmals gezeigt, dass die Hemmung der ATP-Verwertung durch Aktomyosinstrukturen darauf beruht, dass der Bereich der sogenannten überoptimalen Hemmung der ATP-Verwertung sich in Gegenwart des Faktors auf physiologische ATP-Konzentrationen ausdehnt.

Wir zeigen nun, dass das Zusammenwirken von ATP und Faktor ausschliesslich darauf beruht, dass in Gegenwart dieser beiden Komponenten Fibrillen und Aktomyosingele vollständig dissoziiert sind.

1. Nach HUXLEY UND HANSON[24] beruht im physiologischen Ionenmilieu die Dehnung Wasser–Glycerin-extrahierter Muskelfasern auf dem Abgleiten der I-Filamente von den A-Filamenten der Fibrille. Infolgedessen ist der Widerstand gegen die Dehnung ein Masz für die Affinität und Komplexbildung zwischen Aktin- und L-Myosin-Filamenten. Dieser Dehnungswiderstand sinkt bei gleichzeitiger Anwesenheit von ATP und hemmenden Faktormengen auf Null[22,25].

2. Wenn die Affinität zwischen Aktin- und Myosin-Filamenten in Fibrillen oder zwischen Aktin- und Myosin-Molekülen in geordneten Aktomyosinfäden verschwunden ist, ist eine Kontraktion nach dem Schema von HUXLEY UND HANSON[24] unmöglich.

3. In Gegenwart von ATP und Erschlaffungsfaktor wird aus der Aktomyosin-ATPase eine L-Myosin-ATPase. Die Magnesium-aktivierte ATP-Spaltung durch Fibrillen wie durch Aktomyosinflocken fällt in Gegenwart des Faktors nie auf Null. Es bleibt vielmehr immer eine Restspaltung übrig, die in ihrer Grösze der ATP-Spaltung durch L-Myosin in Gegenwart von Magnesium entspricht (Fig. 8).

4. Auf der anderen Seite übt der Faktor auf die beiden Komponenten Aktin und L-Myosin keine Wirkung aus, sobald sie einzeln untersucht werden. Die ATP-Spaltung durch die L-Myosin-ATPase ist von der Faktorkonzentration völlig unabhängig und verläuft mit und ohne Faktor gleich (Tabelle XII). Die Polymerisation von G-Aktin zu F-Aktin (vergl. Spalte 2 mit 1 der Tabelle XIII) und die Viskosität von F-Aktin-

Lösungen (vergl. Spalte 3 mit 1 der Tabelle XIII) werden durch Faktor nicht beeinflusst.

Die Beobachtungen 1–3 zeigen, dass der Faktor in ATP-Gegenwart jede Interaktion zwischen Aktin und L-Myosin aufhebt, während die Beobachtung 4 ergibt, dass er darüber hinaus keine anderen Wirkungen hat. Er ist ein reiner Interaktions-Inhibitor und gehört damit zu einer Reihe von Substanzen, die ausser dem Erschlaffungsfaktor auch noch eine Anzahl anderer chemisch bekannter Stoffe enthält, deren Wirkung in unserem Laboratorium in jüngster Zeit systematisch analysiert und als Wirkung der Interaktions-Inhibitoren zusammengefasst wurde[25,26].

TABELLE XII

GRANAWIRKUNG AUF DIE ATP-SPALTUNG DURCH L-MYOSINFLOCKEN

Grana-Fibrillen Proportion Q	*Spaltungsrate in μMol P/mg L-Myosin · Min*
0	0.016
1	0.016
2	0.013
4	0.016
6	0.017

TABELLE XIII

FEHLENDE WIRKUNG DER GRANA AUF G- UND F-AKTIN

η_{rel} nach Polymerisation einer G-Aktin-Lösung		
	Nach Zugabe von Grana	
Ohne Grana	*Vor der Polymerisation*	*Nach der Polymerisation*
2.6	2.7	2.7
2.1	2.3	2.3
2.4	2.6	2.7

TECHNISCHER TEIL

Zur Gewinnung der Grana wurde die frische Kaninchenmuskulatur zunächst in Eis aus destilliertem Wasser gekühlt und mit dem Fleischwolf zerkleinert. Dann wurde ein Teil Muskelbrei bei 0° mit vier Teilen einer Lösung, die 0.1 *M* KCl, 0.005 *M* Oxalat und 0.005 *M* Phosphat- oder Histidinpuffer enthält, 2 Min lang im Starmix zerkleinert. Zur Abtrennung der Myofibrillen und der Mitochondrien wurde der Brei 20 Min lang bei 8000 × *g* zentrifugiert. Aus dem Überstand (Rohextrakt) wurden die Grana durch einstündiges Zentrifugieren bei 25,000 × *g* niedergeschlagen. Der Graniederschlag wurde in einer Lösung, die 0.1 *M* KCl und 0.001 *M* Oxalat enthält, aufgeschwemmt und mit einem Homogenisator suspendiert. Einige Präparationen wurden zur Konservierung mit 50 Volumenteilen 90 % Glycerin versetzt.

Einige Myofibrillensuspensionen enthielten nach der üblichen Präparation[27] noch spaltungs- und kontraktionshemmende Grana. Zur Inaktivierung der Grana wurde die Fibrillensuspension mit Thymol gesättigt. Überschüssiges Thymol wurde durch gründliches Waschen mit 0.1 *M* KCl entfernt.

L-Myosin wurde nach PORTZEHL, SCHRAMM UND WEBER[28] und Aktin nach den Angaben von STRAUB[29] präpariert.

Die ATP-Spaltung durch Myofibrillen wurde in den Ansätzen gemessen, die 0.055 M KCl, 0.005 M ATP, 0.005 M Mg^{++}, 0.003 M Oxalat, 0.01 M Histidin (pH 7.0) und 0.03–0.05 % Fibrillenprotein enthielten. In den Spaltungsansätzen zur Messung der ATPase-Aktivität von L-Myosinflocken betrug die KCl-Konzentration nur 0.03–0.04 M bei sonst gleicher ionaler Zusammensetzung und gleicher Proteinkonzentration. Die Spaltungsexperimente wurden bei Zimmertemperatur (20–23°) durchgeführt. Abweichungen von diesen Angaben sind in den Legenden vermerkt. Zur Messung der Spaltungsraten wurden den Versuchsansätzen in bestimmten Zeitabständen aliquote Teile entnommen und in Trichloressigsäure (Endkonzentration 1 %) pipettiert. Wenn in den Legenden nicht anders vermerkt, wurde das zwischen der 2. und 7. Min nach Komplettierung der Ansätze freigesetzte Phosphat gemessen. Phosphatbestimmung: nach ROCKSTEIN UND HARRON[30].

Zur Entfernung des Calciums aus kristallisiertem Trypsin wurde das Trypsin in 1 % Lösung 10 Stunden gegen Dowex 50 × 8·Na (pH, 7.0) dialysiert. Die Trypsinverdauung der Grana wurde durch Zugabe von Trypsin Inhibitor (Trypsin:Inhibitor = 1:2) beendet.

Calcium wurde komplexometrisch mit EDTA und Murexid als Indikator bestimmt.

NACHTRAG BEI DER KORREKTUR

Die Eigenspaltung der Grana beträgt 0.1 bis 0.2 µMol ATP/mg Pr.·Min. Sie ist in den Experimenten abgezogen worden.

(Eingegangen am 28 März, 1960)

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ZUSAMMENFASSUNG

1. Die spaltungs- und kontraktionshemmende Granafraktion des Kaninchenmuskels wird aus dem Rohextrakt durch einstündiges Zentrifugieren bei 25,000 × g praktisch vollständig niedergeschlagen. Der Durchmesser der Grana dieser Fraktion beträgt etwa 1000 Å (700 Å–3000 Å).

2. Die Granawirkung wird irreparabel zerstört durch SH-Reagenzien, Thymol und u.v.-Licht. Die Funktionslähmung durch manche Detergentien, wie Antarox, ist teilweise reparabel.

3. Verdauung der Grana mit dem mehrfachen Eigengewicht an Trypsin zerstört die Granawirkung irreversibel in Abwesenheit von ATP und beeinflusst die Wirkung gar nicht in Anwesenheit von ATP.

4. Bevor die Grana hemmend wirken müssen sie in Abwesenheit von Calcium durch ATP und Magnesium in einen aktiven Zustand gebracht werden. Die Aktivierung dauert wenige Minuten und findet in den üblichen Versuchen während des Versuches selbst statt.

5. Die spaltungs- und kontraktionshemmende Wirkung der Grana auf Fibrillen und Aktomyosinflöckchen hört mit der Entfernung der Grana sofort auf.

6. Die Grana entziehen also den kontraktilen Proteinen keine kontraktionswichtige Substanz und geben auch keine stabile kontraktionshemmende Substanz an das Aktomyosin oder die Lösung ab.

7. In einem grossen Konzentrationsbereich ist die Wirkung der Grana nicht von ihrer absoluten Konzentration, sondern von der Proportion der Grana zu den Fibrillen abhängig, obwohl die Grana von den Fibrillen nicht gebunden werden. Infolgedessen können die Grana nicht mit dem Inhibitor identisch sein, der mit den Aktin- und Myosinfilamenten unmittelbar reagiert.

8. Dagegen wird die Abgabe eines labilen Inhibitors durch die Grana wahrscheinlich, durch die Beobachtung, dass die Granawirkung durch mehrmaliges Waschen mit Fibrillensuspensionen erschöpft werden kann. Die Fibrillen entfernen bei diesen Waschungen keine Grana, sondern nur ihre Wirkung.

9. Es wird gezeigt, dass die Grana auf Fasern und andere Aktomyosinsysteme allein dadurch wirken, dass sie bei Anwesenheit von ATP eine vollständige Dissoziation der Aktin- und Myosinfilamente oder -Moleküle herbeiführen. Die L-Myosin-ATPase, sowie der Polymerisationszustand und die Polymerisationsfähigkeit des Aktin werden durch Erschlaffungsfaktor nicht beeinflusst.

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DIE ABHÄNGIGKEIT DER GRANAWIRKUNG VON DER ART DES AKTOMYOSINSYSTEMS UND GERGELY'S CO-FACTOR

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SUMMARY

The dependence of granule effect on the nature of the actomyosin system and Gergely's co-factor

1. Granules inhibit the ATP-cleavage and superprecipitation of Mg-activated single fibrils, actomyosin floccules and very thin actomyosin threads. Inhibition stops when the granules are removed.

2. Extracted single fibres are completely inhibited by their intrinsic granule system, if extraction has not exceeded 1–2 days. Such fresh fibres contract immediately upon addition of Ca.

3. The contraction of fibres that have been extracted over somewhat longer time can be suppressed by the addition of granules in only about 30 % of the cases. After removal of the granules, contraction sets in anew.

4. Inhibition becomes total if in addition to the granules GERGELY's co-factor is added. After the removal of the granules and co-factor, contraction sets in again in only 60 % of the cases.

5. Where the intrinsic granules in the extracted fibres have been irreversibly destroyed by Salyrgan or excessively long extraction, contraction cannot be inhibited longer even by added granules plus co-factor. Similarly, the contraction of thick actomyosin threads cannot be inhibited either by granules alone, or by granules plus the co-factor.

6. The contraction of thick actomyosin threads, however, is at once inhibited if the granules are embedded in the threads instead of being added to the bath. The ATP-splitting produced by thick (100 μ) microtome sections is not affected by the granules; at the moment, however, when the microtome sections have been minced by an homogenizer the splitting is inhibited.

7. All experiments with myosin particles of different dispersity, or with single fibres whose intrinsic granules have been destroyed, support the conclusion that the granules do not work when the diffusion paths of the relaxing substance become long. This is a fresh argument in favour of the assumption of a labile relaxing agent.

8. The effect of added granules plus co-factor on fibres whose intrinsic granule system has been damaged but not irreversibly destroyed, seems to indicate that besides releasing the relaxing agent, granules and co-factor release—or, in the case of the co-factor, are themselves—substances that restore the intrinsic granules of the fibre.

EINLEITUNG

Gereinigte Granasuspensionen verwandeln immer die Aktomyosin-ATPase isolierter Fibrillen in die L-Myosin-ATPase und verhindern die Fibrillenkontraktion, falls ATP und Magnesium zugegen sind[1]. Die für die Wirkung notwendigen Q-Werte* sind gut reproduzierbar. Mit den gleichen Q-Werten werden die gleichen Effekte mit feinen Flocken gereinigten Aktomyosins erzielt (Fig. 1, Tabelle III). (Über weitere Beobachtungen an Aktomyosinflocken und Granawirkung vergleiche die ausführliche Arbeit von Mueller[2].)

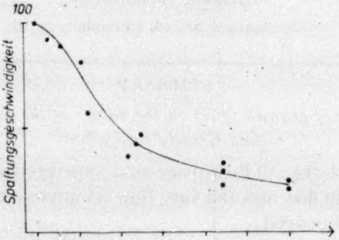

Fig. 1. Die Abhängigkeit der ATP-Spaltung durch Aktomyosinflocken von der Proportion des Grana-N zum Aktomyosin-N. Ordinate: Geschwindigkeit der Spaltung des ATP durch Aktomyosin, ungehemmte Spaltung = 100. Abszisse: Q-Werte = Grana N·Aktomyosin N^{-1}·100. Mg^{++} = ATP = Oxalat = $2.5 \cdot 10^{-3}$ *M*.

Auf isolierte extrahierte Fasern und Faserbündel wirken gereinigte Suspensionen der Grana in normalem Umfange nur in einem gewissen Prozentsatz der Versuche (in 8 von 26 Experimenten). (Über die häufige Wirkungslosigkeit der Grana allein vergl. auch die Arbeit von Briggs *et al.*[3].)

Wie Briggs *et al.*[3] gezeigt haben, wirken die Grana viel häufiger hemmend, wenn zu den Fasern ausser der Granasuspension auch der von den genannten Autoren entdeckte Cofaktor hinzugefügt wird. Wir erhalten mit Cofaktor Hemmung in 44 von 52 Experimenten.

Der Rest der Fasern (8 von 52) erschlafft selbst in Gegenwart von Grana und Cofaktor nicht. Er ist doppelt so lange gealtert wie die erschlaffungsfähigen Fasern (zwei Monate, davon einige Tage bei Zimmertemperatur).

Wenn die Granawirkung nicht an feinen Aktomyosinflöckchen, sondern an kontraktilen, wohlgeordneten Aktomyosinfäden nach Portzehl[4] (16 Versuche) oder an ungeordneten, unter ATP schrumpfenden Fadenstückchen nach A. Szent-Györgyi[5] (18 Versuche) geprüft wird, so zeigt sich, dass die Kontraktion und Schrumpfung weder durch Grana allein, noch durch Grana zusammen mit Cofaktor gehemmt wird.

Wenn die gereinigte Granasuspension von den extrahierten Fasern oder Faserbündeln entfernt wird, tritt in allen Fällen eine neue Kontraktion ein, in denen die Granasuspension allein vorher erschlaffend gewirkt hat. Unter diesen Bedingungen wird die Hemmung der Fasern durch die Granaentfernung ebenso beendet wie die Hemmung isolierter Fibrillen. Wenn dagegen eine Erschlaffung durch das Zusammenwirken von Grana und Cofaktor hervorgerufen war, überdauert die Erschlaffung ganzer extrahierter Fasern die Entfernung der Grana in etwa 50 % der Experimente (20 von 38) und die Entfernung von Grana und Cofaktor in einer beträchtlichen Zahl von Experimenten (13 von 34).

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* Q = Grana-N·Fibrillen-N^{-1}·100.

Zusammenfassend sagen diese Beobachtungen folgendes: (a) Wenn die Diffusionswege innerhalb der geprüften Partikel lang sind (Fasern, Faserbündel, Bruchstücke von Aktomyosinfäden) fehlt die erschlaffende Wirkung der Grana oft (extrahierte Fasern) oder immer (Aktomyosinfäden und ihre Bruchstücke). (b) Auf der anderen Seite überdauert die Erschlaffung von Fasern und Faserbündel, die durch das System Grana und Cofaktor hervorgerufen ist, häufig die Entfernung der Grana und nicht selten sogar die Entfernung von Grana und Cofaktor.

I

Die Unzuverlässigkeit und das Fehlen der erschlaffenden Wirkung der Grana scheint nicht auf chemischen Verschiedenheiten zwischen den grob dispersen Partikeln (Aktomyosinfäden und Fasern) und den fein dispersen Partikeln (Aktomyosinflocken und Fibrillen) zu beruhen, sondern auf der verschiedenen Dispersität. Die ATP-Spaltung durch 100 μ dicke Gefrierschnitte von extrahierter Muskulatur wird nämlich durch gereinigte Granasuspensionen selbst dann nicht oder nur geringfügig beeinflusst, wenn die Proportion der Grana zu den Schnitten sehr hoch ist. Die Hemmung setzt dagegen sofort ein, wenn in der gleichen Lösung die gleichen Schnitte mechanisch in feine Teilchen zerlegt werden (Tabelle I). Ebenso wird aus der ungehemmten Kontraktion und Schrumpfung der geordneten Aktomyosinfäden nach PORTZEHL[4] und der groben Fadenbruchstücke nach A. SZENT-GYÖRGYI[5] volle Hemmung von Superpräcipitation und ATP-Spaltung, wenn aus der gleichen Aktomyosin-Lösung feine Flöckchen hergestellt werden (vergl. Tabelle III).

TABELLE I

ABHÄNGIGKEIT DER GRANAWIRKUNG AUF DIE AKTOMYOSIN-ATPASE VOM DISPERSITÄTSGRAD DES EXTRAHIERTEN MUSKELGEWEBES

Die ATP-Spaltung in Prozent der Spaltung ohne Grana			
Durch ganze Schnitte		*Durch zerkleinerte Schnitte*	
Ohne Grana	*Mit Grana**	*Ohne Grana*	*Mit Grana**
100	100	100	52
100	100	100	38
100	80	—	—
100	78	—	—

* Q, ~ 10.

Man kann sogar die Schrumpfung 400 μ dicker Fadenbruchstücke nach SZENT-GYÖRGYI fast vollständig aufheben, wenn die Grana nicht in einer Suspension von aussen zugegeben werden, sondern bei der Herstellung der Fäden in die Grana eingelagert werden (Tabelle II)*. Eine eben erkennbare Anfangsschrumpfung der Faden-

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* Granahaltige geordnete Fäden nach PORTZEHL[4] konnten nicht hergestellt werden, weil die granahaltigen Fäden zu brüchig sind.

TABELLE II

DER EINFLUSS DER GRANA AUF DIE SCHRUMPFUNG VERSCHIEDENER AKTOMYOSINPRÄPARATE

Art der Präparation Proteingehalt der Fäden: 0.5% Q = 10–20	*Schrumpfung in Prozenten des Anfangsvolumens*					
	Ohne Grana		*Grana aussen*		*Grana innen*	
	Ohne Cofaktor	*Mit Cofaktor*	*Ohne Cofaktor*	*Mit Cofaktor*	*Ohne Cofaktor*	*Mit Cofaktor*
Flocken	85	—	0 (12)*	—	—	—
Fäden: 2r = $\gtrsim$ 800 μ	85 (6)	85 (12)	85 (9)	85 (12)	60 (9)	60 (11)
Fäden: 2r = $\gtrsim$ 400 μ	85 (8)	85 (18)	85 (7)	85 (10)	20 (10)	20 (10)

* (..): Anzahl der Versuche.

bruchstücke nach Szent-Györgyi[5] ist allerdings unvermeidbar, weil die Grana nur dann hemmen, wenn gleichzeitig genügend ATP zugegen ist. In dicken Fadenstücken nach Szent-Györgyi[5] dauert es aber eine gewisse Zeit, bis die ATP-Konzentration im Inneren von Null auf den Betrag angestiegen ist, der für die Granahemmung notwendig ist. Der Betrag dieser Anfangsschrumpfung wächst mit der Diffusionszeit der ATP, d.h. mit der Dicke der Fadenbruchstücke (Tabelle II).

TABELLE III

DER EINFLUSS DER GRANA AUF DIE ATP-SPALTUNG VERSCHIEDENER AKTOMYOSINPRÄPARATE

Art der Präparation Proteingehalt der Fäden: 2–3% Q = 10–40	*Geschwindigkeit der ATP-Spaltung μMol P/mg Protein · Min*							
	Ohne Grana		*Grana aussen*		*Grana innen*			
							Zusätzl. Grana aussen	
	Ohne Cofaktor	*Mit Cofaktor*	*Ohne Cofaktor*	*Mit Cofaktor*	*Ohne Cofaktor*	*Mit Cofaktor*	*Ohne Cofaktor*	*Mit Cofaktor*
Flocken	0.40 (10)*	0.40 (5)	0.06 (12)	0.06 (12)	—	—	—	—
Fäden: 2r = 200 μ	0.36 (12)	0.36 (12)	0.15 (10)	0.15 (10)	0.15 (12)	0.13 (12)	0.10 (14)	0.08 (14)
Fäden: 2r = 600 μ	0.20 (10)	0.20 (6)	0.15 (10)	0.14 (8)	0.20 (10)	0.14 (6)	0.10 (12)	0.08 (6)

* (..): Anzahl der Versuche.

Tabelle III zeigt die Hemmung der ATP-Spaltung durch Aktomyosinflocken und ungeordnete Aktomyosinfäden unter dem Einfluss von Grana. Die ATP-Spaltung durch Aktomyosinflöckchen wird durch Grana genau so gehemmt, wie die Superpräcipitation der Flöckchen. Auch die ATP-Spaltung durch sehr dünne ungeordnete Fäden niedrigen Eiweissgehaltes (2–3 %) wird durch Grana "aussen" voll gehemmt. Wenn die Aktomyosinfäden dagegen sehr dick sind, lässt sich nicht entscheiden, wie weit die Grana die ATP-Spaltung hemmen. Denn dicke Fäden spalten auch in Abwesenheit von Grana so schlecht (Tabelle III, Reihe 3), dass eine weitere Herabsetzung der Spaltungsgeschwindigkeit durch Granaeinfluss nicht mehr sicher messbar ist. Diese geringe Spaltungsrate beruht darauf, dass die dicken Fäden dreimal so dick sind wie die Warburg-Meyerhofsche Grenzschichtdicke*. Infolgedessen wird ohne

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* Die Grenzschichtdicke von Fäden mit einem Aktomyosingehalt von 2–3 % ist mit Hilfe der Diffusionskonstanten $D = 8\text{–}10 \cdot 10^{-7}$ cm^2·sec^{-1} berechnet. Diese Diffusionskonstante ergibt sich aus dem Vergleich der ATP-Spaltung von Flocken, dünnen Fäden und dicken Fäden, in Abwesenheit von Grana (vgl. TECHNISCHER TEIL).

Grana das ATP in den Randschichten ungehemmt und in den tieferen Schichten gar nicht oder kaum gespalten. Es ist deshalb zu erwarten: (a) Eine Spaltungshemmung durch Grana setzt die Spaltungsgeschwindigkeit in den Randschichten herab. Die Spaltungsgeschwindigkeit wird nicht null, weil die Grana die Aktomyosin-ATPase in die Ł-Myosin-ATPase verwandelt[1]. (b) Infolge der Herabsetzung der Spaltung dringt das ATP tiefer in das Fadeninnere ein. (c) Dieses tiefer eindringende ATP wird in dem vorher ATP-freien Kern der Fäden doch noch gespalten. Denn die Spaltung von sehr verdünntem ATP wird durch Grana nicht gehemmt.

Die Abnahme der ATP-Spaltung in den Aussenschichten unter dem Einfluss der Grana wird infolgedessen durch eine ATP-Spaltung in den vorher ATP-freien Innenschichten mehr oder minder kompensiert.

Die Anwesenheit des Cofaktors erhöht den Einfluss der Grana auf die ATP-Spaltung durch gereinigte Flocken und Fäden ebensowenig wie die Hemmung von Superpräcipitation und Schrumpfung.

II

Eine Aufhebung der Granawirkung und nicht nur eine Verzögerung durch lange Diffusionswege ist zu erwarten, wenn die von den Grana abgegebene eigentliche Erschlaffungssubstanz labil ist[1]. Denn dann sollte sie schon auf dem Diffusionswege zerstört werden. Es liegt dann nahe anzunehmen, dass der Cofaktor von GERGELY[6] und BRIGGS[3] die Granasuspensionen auch gegenüber extrahierten Fasern und Muskelschnitten deshalb wirksam macht, weil er die labile Erschlaffungssubstanz stabilisiert. Wenn diese Annahme richtig ist, sollte die Hemmung der ATP-Spaltung durch Fibrillen in Anwesenheit von Cofaktor die Entfernung der Grana überdauern. Das Ergebnis eines solchen Versuches ist von WEBER unter Hinweis auf diese Arbeit bereits 1958 in New York veröffentlicht worden[7,8]. In der Cofaktor-freien Lösung hörte die Granawirkung mit der Granaentfernung auf, während bei dauernder Anwesenheit des Cofaktors die Hemmung durch die Grana die Granaentfernung für eine begrenzte, aber gut messbare Zeit überdauerte. Hieraus schloss WEBER, dass der Cofaktor die Lebensdauer der labilen Erschlaffungssubstanz verlängert.

Dieser Schluss hat aus drei Gründen viel von seiner Beweiskraft verloren.

1. Hat es sich bei einer grösseren Versuchszahl herausgestellt, dass die geschilderten Ergebnisse nur in 25 % der Versuche beobachtet werden. In den übrigen 75 % hört die Granawirkung in Anwesenheit und Abwesenheit des Cofaktor mit der Granaentfernung auf. Dieses Zahlenverhältnis weckt den Verdacht, dass in einigen Experimenten die Entfernung der Grana weniger vollständig war, als es geplant war.

2. Wenn der Cofaktor durch Stabilisierung auch das Innere grob disperser Partikel für die sonst labile Erschlaffungssubstanz zugänglich macht, müssen in Anwesenheit von Cofaktor und Grana nicht nur die extrahierten Fasern, sondern auch die Aktomyosinfäden gehemmt werden. Aktomyosinfäden genügender Dicke und ihre Bruchstücke aber werden durch Grana "aussen" weder in Anwesenheit noch in Abwesenheit von Cofaktor gehemmt (Tabelle III). Als genügend dick werden Fäden bezeichnet, in denen das Verhältnis des Durchmessers zur Grenzschichtdicke für ATP etwa 3–4:1 beträgt.

3. Die hemmende Wirkung einer "guten" Granasuspension auf die ATP-Spaltung durch Fibrillen wird durch die Anwesenheit von Cofaktor nicht erhöht. Die Hemmung

wird auch dann nicht erhöht, wenn die Proportion der "guten" Grana zu den Fibrillen so klein gewählt wird, dass mit und ohne Cofaktor nur eine unvollständige Hemmung eintritt. Die Hemmung müsste aber durch Akkumulation der Erschlaffungssubstanz erhöht werden, wenn die Lebensdauer der von den Grana gelieferten Erschlaffungssubstanz in Gegenwart des Cofaktors gröszer ist als in seiner Abwesenheit (Fig. 2) (vergl. auch GERGELY *et al.*[6]).

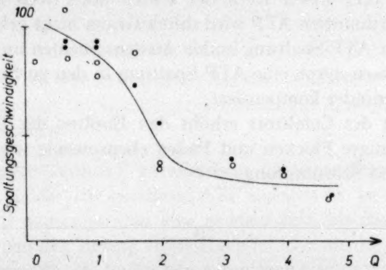

Fig. 2. Der fehlende Einfluss des Cofaktors auf die Hemmung der Fibrillen-ATPase durch ungeschädigte Grana. Ordinate: Geschwindigkeit der ATP-Spaltung durch Fibrillen in Prozenten der ungehemmten Spaltung. Abszisse: Q-Werte. Gefüllte Symbole: ohne Cofaktor. Offene Symbole: mit Cofaktor.

Aus allen diesen Gründen muss bezweifelt werden, dass eine Stabilisierung der Erschlaffungssubstanz durch den Cofaktor für die Wirkung des Faktors entscheidend ist.

Offenbar wird aber auch die Abgabe der Erschlaffungssubstanz seitens der Grana durch den Cofaktor von GERGELY[6] und BRIGGS[3] nicht aktiviert. Denn eine solche Aktivierung müsste sich (genauso wie eine Stabilisierung der labilen Erschlaffungssubstanz) in einer Erhöhung der hemmenden Wirkung der Grana auf die ATP-Verwertung durch Fibrillen bemerkbar machen. Dagegen haben GERGELY *et al.*[6] die verminderte Wirkung geschädigter Grana fast vollständig restituiert, wenn sie der Grana-Fibrillen-Suspension Cofaktor zufügten.

Es liegt also auf Grund der Fibrillenversuche nahe anzunehmen, dass der Cofaktor weder stabilisierend auf die Erschlaffungssubstanz noch aktivierend auf normale Grana, aber reaktivierend auf geschädigte Grana wirkt.

III

Für das Zusammenspiel von Grana, labiler Erschlaffungssubstanz und Cofaktor gegenüber Wasser-Glyzerin-extrahierten Faserbündeln scheint sich aus den Ergebnissen der letzten beiden Abschnitte folgendes Bild zu ergeben.

1. Die granaproduzierte labile Erschlaffungssubstanz ist anscheinend unfähig, im Inneren von Aktomyosinsystemen lange Diffusionswege zurückzulegen. Deshalb wirkt sie weder in Anwesenheit noch in Abwesenheit des Cofaktors auf granafreie Aktomyosinfäden, genügender Dicke.

2. In extrahierten Muskelfasern ist das Granasystem vorhanden. Dieses bodenständige Granasystem ist voll aktionsfähig, wenn die Fasern sehr frisch sind. Infolge-

dessen kontrahieren sich sehr frisch extrahierte Fasern in der Regel nur dann, wenn ausser dem ATP noch soviel Calcium zugesetzt wird, dass das bodenständige Granasystem durch dieses Calcium inaktiviert wird.

Wenn die Fasern sehr stark gealtert sind (~ zwei Monate unter Einschaltung einiger Tage bei Zimmertemperatur), ist das bodenständige Granasystem in der Regel vollständig und irreversibel funktionsunfähig. In diesem Falle erschlaffen die Fasern ebenso wenig in einer Cofaktor-haltigen Granasuspension wie die Aktomyosinfäden*. Im mittleren Stadium der Alterung dürfte das bodenständige Granasystem in einem mehr oder minder reversiblen Zustand der Schädigung sein. In diesem Stadium werden die Fasern nicht selten durch die Granasuspension allein und sehr oft durch Grana und Cofaktor gehemmt.

Es liegt infolgedessen nahe, die Erschlaffung extrahierter Fasern mittleren Alters auf eine Reaktivierung des fasereigenen Granasystems zu beziehen. Denn (a) ist die Reaktivierung geschädigter Grana die einzige Wirkung des Cofaktors, die bisher nachgewiesen werden konnte[6]; (b) wird die Schrumpfung "granafreier" Aktomyosinfäden durch Zugabe von Grana und Cofaktor nicht gehemmt.

Da Fasern mittleren Alters aber nicht selten durch Grana allein gehemmt werden, müssen wir schliessen, dass auch die Grana eine Reaktivierungssubstanz abgeben, die ähnlich wie der ebenfalls reaktivierende Cofaktor in der Lage ist, tief in die Fasern bis zu den bodenständigen Grana vorzudringen. Es ist möglich, dass die labile Erschlaffungssubstanz und die vermutete reaktivierende Substanz von verschiedenen Fraktionen der Granasuspension abgegeben werden.

TECHNISCHER TEIL

Aktomyosin wurde nach PORTZEHL, SCHRAMM UND WEBER[9] präpariert. Um ein konzentriertes Aktomyosin-Gel zu erhalten, wurde das dreimal umgefällte Aktomyosin bei einer Ionenstärke von 0.1 μ durch Zentrifugieren (40,000 × g, 30 Min) niedergeschlagen. Durch Zusatz von festem KCl wurde die Ionenstärke des 4–5 % Aktomyosin-Gel auf 1.0 μ erhöht.

Die Grana-freien und die Grana-haltigen Aktomyosinfäden wurden aus Aktomyosin-Solen gleicher Aktomyosinkonzentration (2–3 %) hergestellt. Diese Aktomyosin-Sole wurden durch Zusatz von 0.1 M KCl, bzw. einer Granasuspension in 0.1 M KCl, zu dem 4–5 % Aktomyosin-Sol erhalten. Die Durchmischung des konzentrierten Aktomyosin-Sol mit der 0.1 M KCl-Lösung, bzw. mit der Granasuspension, erfolgte in einem Polyaethylen-Röhrchen, an dessen Innenwand ein langsam rotierende Plexiglasstab fest angepresst war. Die in das Sol eingeschlossenen Luftblasen wurden durch Zentrifugieren entfernt. Je 0.1–0.15 ml des Aktomyosin wurden mit Agla-Mikrometerspritzen, deren Glasspitzen zu Kapillaren (200 μ und 600 μ) ausgezogen waren, in die ATP-freien Spaltungsansätze gespritzt[1].

Zur Bestimmung der Spaltungsgeschwindigkeit wurde die Spaltung im ersten Ansatz 2 Min nach ATP-Zugabe und in einem zweiten Ansatz gleicher Zusammensetzung 7 Min oder 12 Min nach ATP-Zugabe durch Zusatz von 5 % TCA unterbrochen. Alle Spaltungsexperimente wurden als Doppelbestimmungen durchgeführt. Neben der Spaltung des ATP durch die Fäden wurde in allen Experimenten auch die Spaltung des ATP durch feine Aktomyosinflocken geprüft, die durch mechanische Zerkleinerung der Grana-freien und Grana-haltigen Fäden erhalten wurden.

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* Dass diese Resistenz sehr alter Fasern nicht auf einer Veränderung des eigentlich kontraktilen Systems beruht, lässt sich durch Zusatz chemischer Interaktionsinhibitoren zeigen. Auf Zusatz der diffusiblen chemischen Interaktionsinhibitoren (Mg-EDTA, Polyaethylensulfonsäure) erschlaffen auch die sehr alten Fasern sofort.

Phosphat wurde nach ROCKSTEIN UND HARRON[10] bestimmt. In gesonderten Experimenten wurde der Durchmesser der Fäden vor und nach ATP-Einwirkung gemessen. Der Durchmesser der Fäden mit einem Proteingehalt von 2–3 % nimmt unter ATP-Einwirkung praktisch nicht ab.

Die lockeren Aktomyosinfäden nach SZENT-GYÖRGYI[5] wurden aus 0.5 % Aktomyosinlösungen hergestellt. Ihre Schrumpfung wurde mit dem Mikroskop beobachtet.

Die geordneten Aktomyosinfäden, an denen Spannungsmessungen vorgenommen wurden, wurden nach PORTZEHL[4] hergestellt. Die Spannungsentwicklung der geordneten Aktomyosinfäden und der extrahierten Psoasfasern wurde mit dem Dilatometer nach WEBER[11] gemessen.

Die Diffusionskonstante des ATP in den Aktomyosinfäden wurde mit Hilfe der Meyerhof-schen Formel[12] und der von HILL[13] angegebenen Gleichung bestimmt.

Die 100 μ dicken Muskelschnitte wurden nach Extraktion der Faserbündel in 0.1 M KCl mit dem Gefriermikrotom hergestellt. Die Schnitte wurden mit groszem Volumen 0.1 M KCl-Lösung (pH 7.0 Histidinpuffer) wiederholt gewaschen. Zur Bestimmung der ATP-Spaltung wurden in einen 20-ml-Ansatz 5–10 Schnitte gegeben. Ein feinmaschiges Kunststoffsieb verhinderte, dass beim Herauspipettieren aliquoter Anteile des Ansatzes die Schnitte aus dem Spaltungsansatz mitentfernt wurden. In einer Reihe von Versuchen wurden nach Messung der Spaltungsaktivität der Schnitte diese Schnitte in dem gleichen Spaltungsansatz schnell mit dem Homogenisator zerkleinert und anschliessend die Spaltungsaktivität der zerkleinerten Schnitte bestimmt.

Zur Präparation der Grana und zur Messung der Geschwindigkeit der Spaltung des ATP durch Aktomyosinflocken und Fibrillen siehe die vorangegangene Mitteilung[1].

Proteinfreie Kofaktorlösungen werden durch 12–18-stündige Dialyse des Grana-Rohextraktes vor oder nach Entfernung der Grana gegen ein kleines Volumen einer Lösung von 0.1 M KCl hergestellt.

ANHANG

Werden die bodenständigen Grana, die in allen extrahierten Fasern vorhanden sind, durch Salyrgan* irreversibel vergiftet, so kontrahieren sich die Fasern noch völlig normal, sobald das Salyrgan ausgewaschen ist. Dieser kontrahierte Zustand lässt sich nun aber nicht mehr dadurch aufheben, dass hochwirksame Grana + Cofaktor dem Bad in derselben Konzentration zugesetzt werden, in der sie vor der Vergiftung voll wirksam waren (Fig. 3). Grana + Cofaktor im Bad wirken also auf extrahierte Fasern, deren eigene bodenständige Grana zerstört sind, genausowenig wie auf dicke Aktomyosinfäden, die keine bodenständigen Grana enthalten.

Damit sind durch direkten Beweis die Schlussfolgerungen unserer Arbeit bestätigt: (a) Das Erschlaffungssystem produziert eine Erschlaffungssubstanz, die in Fibrillen und Aktomyosinflocken die Interaktion zwischen Myosin und Aktin unmittelbar aufhebt. Diese Substanz vermag keine langen Diffusionswege zurückzulegen. (b) Das Erschlaffungssystem produziert Substanzen, die funktionsunfähige, aber noch nicht zu sehr geschädigte Grana in extrahierten Fasern reaktivieren, weil sie die dafür notwendigen langen Diffusionswege zurückzulegen vermögen.

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* Salyrgan = Mersalyl, Salicyl (α-hydroxymercuri-β-methoxypropyl) amidoacetat.

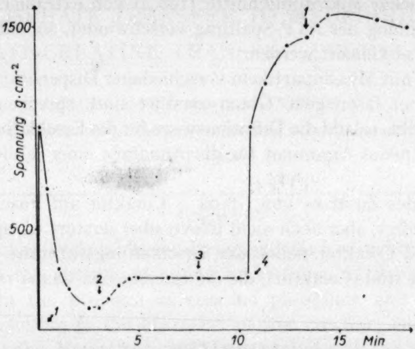

Fig. 3. Die Abhängigkeit der Erschlaffungswirkung des Systems Grana + Cofaktor "aussen" von der Reaktivierbarkeit der fasereignen Grana. Ordinate: Spannung g/cm^2. Abszisse: Zeit in Minuten. Die Versuchsbäder enthielten: 1. ATP = Mg^{++} = Oxalat = $5 \cdot 10^{-3}$ *M*; Histidin, 0.01 *M*; pH, 7.0; KCl, 0.05 *M*; Granaprotein, 0.1 %; Cofaktor, 40 % v/v. 2. Salyrgan, $2 \cdot 10^{-4}$ *M*; KCl, 0.1 *M*. 3. Cystein, $5 \cdot 10^{-3}$ *M*; KCl, 0.1 *M*; pH, 7.0. 4. ATP = Mg^{++} = Oxalat = $5 \cdot 10^{-3}$ *M*; Histidin, 0.01 *M*; pH, 7.0; KCl, 0.05 *M*. Temperatur, 22°.

Wir danken Herrn Prof. Dr. H. H. WEBER für anregende Diskussionen, Frau H. VON GRAFF, Fräulein E. GEYRHALTER und Herrn K. J. KLETTI für die Hilfe bei den Experimenten.

ZUSAMMENFASSUNG

1. Die Grana hemmen die ATP-Spaltung und die Superpräcipitation von Magnesium-aktivierten Einzelfibrillen, Aktomyosinflocken und sehr dünnen Aktomyosinfäden. Die Wirkung erlischt mit der Entfernung der Grana.

2. Extrahierte Einzelfasern werden von dem fasereigenen Granasystem vollständig gehemmt, wenn sie nur 1–2 Tage extrahiert sind. Solche frischen Fasern kontrahieren sofort auf Calciumzusatz.

3. Die Kontraktion etwas länger extrahierter Fasern kann nur in etwa 30 % der Fälle durch Zusatz von Grana aufgehoben werden. Auf Entfernung der Grana tritt eine neue Kontraktion ein.

4. Der Erfolg der Hemmung erhöht sich auf 100 %, wenn ausser den Grana auch GERGELYS Cofactor hinzugefügt wird.

Wenn Grana und Cofaktor wieder entfernt werden, tritt nur in 60 % der Fälle eine neue Kontraktion ein.

5. Wenn die fasereigenen Grana im Innern der extrahierten Fasern durch Salyrgan oder sehr lange Extraktion irreversibel zerstört sind, lässt sich die Kontraktion auch durch Grana und Cofaktor nicht mehr hemmen. Ebenso ist die Schrumpfung dicker Aktomyosinfäden weder durch Grana noch durch Grana und Cofaktor hemmbar.

6. Wenn in Aktomyosinfäden einer Dicke, die die Schrumpfung der Fäden gegen Grana "aussen" resistent macht, Grana eingelagert werden, wird die Schrumpfung fast vollständig unterdrückt.

Die Resistenz dicker Mikrotomschnitte (100 μ) von extrahierten Faserbündeln gegen die Granahemmung der ATP-Spaltung verschwindet, sobald die Schnitte mit dem Homogenisator zerkleinert werden.

7. Alle Versuche mit Myosinpartikeln verschiedener Dispersität und die Versuche an Einzelfasern, deren fasereigene Grana zerstört sind, sprechen dafür, dass die Granawirkung ausbleibt, sobald die Diffusionswege für die Erschlaffungssubstanz lang werden. Dies ist ein neues Argument für die Annahme einer labilen Erschlaffungssubstanz.

8. Der Einfluss des Zusatzes von Grana + Cofaktor auf Fasern, deren eigenes Grana-System geschädigt, aber noch nicht irreversibel zerstört ist, spricht dafür, dass zugesetzte Grana und Cofaktor neben der Erschlaffungssubstanz auch Substanzen abgeben (Grana) oder sind (Cofaktor), die die fasereigenen Grana restituieren.

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PHOSPHATE TRANSFER TO MYOFIBRILS BY ATP-CREATINE TRANSPHOSPHORYLASE

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SUMMARY

The following reaction cycle is observed in myofibrils from rabbit muscle treated with deoxycholate: In the absence of added nucleotides, ADP bound to myofibrils is phosphorylated in the presence of creatine phosphate and ATP-creatine transphosphorylase, resulting in the liberation of free creatine, and the ATP produced is hydrolyzed by the Mg-activated ATPase activity of the myofibrils. The same phenomenon also occurs with myofibrils isolated from glycerinated psoas fibers.

INTRODUCTION

Myosin and actin are considered to be the principal proteins involved in muscle contraction[1,2]. Isolated actin always contains tightly bound ADP[3] which can not be removed unless actin is denatured. In spite of much effort, the role of actin-bound ADP in the process of muscle contraction is still obscure[4–7].

STROHMAN[6] has reported that Cr-enzyme could catalyze the reaction between actin-bound ADP and added Cr-P. This transphosphorylation was accompanied by transformation of fibrous to globular actin. PERRY has reported that most of the ADP of myofibrils is bound to actin[4] and that the ADP of myofibrils cannot be phosphorylated[5] under conditions similar to those used by STROHMAN.

The failure to observe transphosphorylation under the conditions used by PERRY, might be explained perhaps by the inability of added Cr-enzyme to penetrate untreated myofibrils. We have found myofibrils treated by deoxycholate or glycerine to have a similar appearance in the microscope to untreated myofibrils. Most of the work reported here has been done on myofibrils treated with deoxycholate. A preliminary report has been previously presented[8].

MATERIALS AND METHODS

Preparation of myofibril

Myofibrils were prepared from rabbit back muscle by the method of PERRY[9], except that 4 mM $MgCl_2$ equal to the concentration of EDTA was added. Such myo-

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Abbreviations: Cr-enzyme, ATP-creatine transphosphorylase (creatine kinase); Pi, inorganic orthophosphate; Cr-P, creatine phosphate; AMP, adenosinemonophosphate; ADP, adenosinediphosphate; ATP, adenosinetriphosphate; EDTA, ethylenediaminetetraacetate; ATPase, adenosinetriphosphatase; *p*-CMB, *p*-chloromercuribenzoate; TCA, trichloroacetic acid.

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fibrils show no Cr-enzyme activity and the sarcomere length is 2.6 to 2.8 μ. If magnesium and EDTA are omitted from the wash solution, the sarcomere lengths are much shorter and more random. The concentration of myofibrils is expressed as protein, determined by the biuret procedure of GORNALL, BARDAWILL AND DAVID[10] using crystalline bovine serum albumin as a standard. Myofibrils were generally stored at 0° as a very thick paste (30 to 35 mg/ml) and portions were weighed for use assuming a density of 1.

Deoxycholate treatment of myofibrils

Commercial deoxycholate was recrystallized twice from methanol. A stock 5 % solution (approx. 0.125 *M*) was prepared after adjusting the pH to 7.8 with KOH. Myofibrils (3 volumes) were first mixed with an appropriate amount of deoxycholate–borate KCl mixture (4 volumes) and thoroughly stirred for 1 min at 0°. Then the mixture was washed 3 times, each with a volume of 0.04 *M* borate–0.03 *M* KCl, pH 7.15, equal to 5 to 6 times the volume of the original myofibril–deoxycholate suspension. The myofibrils were collected each time by centrifugation at 0°, 5000 $\times g$ for 15 min and finally suspended in the borate–KCl solution. Myofibrils treated with deoxycholate were used immediately.

Preparation of myofibrils from glycerinated muscle fibers

Glycerinated fibers were prepared from rabbit psoas muscle as follows: Muscle strands about 2 mm in diameter were partially dissected out and tied *in situ* with cotton thread to glass rods in order to maintain the rest length; the fibers attached to glass rods were allowed to stand at 0° in 50 % glycerine which was changed daily; beginning the third day the fibers in fresh 50 % glycerine at 0° were transferred to a deep freeze at about. 15° and stored for more than two months without change of solution. Glycerinated fibers were cut with scissors to about one-inch length and treated as in the preparation of fresh myofibrils.

Preparation of [^{32}P]creatine phosphate

A mixture of [^{32}P]ADP and [^{32}P]ATP was prepared from radioactive inorganic phosphate using liver mitochondria[11] and the two nucleotides were purified by the procedure of PONTIS[12] using calcium chloride on a Dowex-1 column, but modified for batch operation—0.05 *M* NH_4Cl was used to wash the column, 0.01 *N* HCl to elute AMP and 0.035 *M* $CaCl_2$, pH 1.85, to elute ADP + ATP. [^{32}P]Cr-P was prepared from the calcium-free mixture of labeled adenine nucleotides by incubating 30 min at room temperature at pH 9.5 with excess creatine and purified preparations of Cr-enzyme and myokinase. [^{32}P]Cr-P was easily isolated by ion-exchange chromatography on a Dowex-1 chloride column (1 cm × 3.5 cm) by eluting with 0.025 *M* calcium chloride, pH 4.2. The effluent was lyophilized, the dry powder was dissolved in 1 m*M* NH_4OH and calcium was removed by the use of a Dowex-50 X Na^+-form column. The solution of [^{32}P]Cr-P contained less than 5 % Pi and showed no absorption at 260 mμ. The overall yield from starting compound, $Na_3{}^{32}PO_4$, to [^{32}P]Cr-P was 38 %.

A Nuclear Chicago Geiger tube and scaler were used for monitoring radioactivity and samples were plated and counted using a gas flow counter.

Determination of creatine liberating activity

The reaction mixture was composed of 0.300 ml 0.10 *M* buffer (generally histidine buffer, pH 7.0), 0.100 ml 0.01 M $MgCl_2$, 0.050 ml or more of Cr-enzyme (generally, 45–50 mg crystallized enzyme/ml in 0.001 M glycine, pH 9), myofibril stock (0.100–0.500 ml of myofibril suspension containing about 10 mg/ml in borate–KCl solution; biuret protein determination is made) and 0.04 M borate–0.03 M KCl, pH 7.15, to a total volume of 0.900 ml. The reaction (incubated in 30° bath) was started by adding 0.100 ml Cr-P to a concentration of 1–10 mM and stopped by the addition of 2.5 mM p-CMB to a final concentration of 0.5 mM followed by cooling in an ice-water mixture. Protein was precipitated using barium hydroxide and zinc sulfate as described by SOMOGYI[13]. The supernatant from centrifugation, 5,000 × g, 0°, 15 min, was used for creatine determination by the method of ENNOR AND STOCKEN[14].

Determination of ATPase activity

Incubation at 30° was carried out in the presence of 1 mM ATP, 5 mM $MgCl_2$, 50 mM histidine, pH 7, and the Pi liberated was determined by the method of FISKE AND SUBBAROW[15].

Determination of radioactive phosphate incorporation

Incubation conditions were the same as in the determination of creatine liberation except that 5 times larger quantities were used. The reaction was stopped and the nucleotide liberated by adding cold 3% TCA, and allowing the mixture to stand in an ice bath for 20 min. The mixture was centrifuged at 0° and the precipitate was once washed with 1% TCA. The combined supernatant solutions were adsorbed on a Norite column (25 mg Norite A and 15 mg cellulose powder). To reduce the ^{32}Pi and [^{32}P]Cr-P contamination, the column was washed 3 times with a total of 30 ml of 0.1 M NaH_2PO_4. The Norite A column was transferred to a planchet and dried for counting.

RESULTS

Morphological structure of myofibrils

Myofibrils prepared as described above have been found to be heterogeneous in size, but the sarcomere lengths have been found to be almost uniform. Myofibrils washed with deoxycholate solution showed a morphological structure similar to untreated myofibrils except that the I band was somewhat shorter, the Z membrane was difficult to see even at 1000 × magnification, and the outer boundaries of the myofibrils were less distinct.

At low ionic strength in the absence of Mg^{++}, myofibrils shortened in 5 mM ATP and showed a simple pattern of alternating dark and light bands.

Myofibrils prepared from glycerinated muscle fibers appeared to have a more distinct band pattern than myofibrils prepared from untreated muscle myofibrils.

Content of nucleotides of myofibrils

The nucleotides bound to myofibrils were eluted with 1% $HClO_4$ and analyzed by ion-exchange chromatography on Dowex-1 according to the method of COHN AND CARTER[16] adapted to smaller quantities. Adenine nucleotides were further identified by optical density ratios 250 mμ/260 mμ = 0.22 and 280 mμ/260 mμ = 0.85. As seen in Table I the amounts of the nucleotides found were slightly lower than those reported by PERRY[4] (determined by a combination of phosphate analysis and enzyme assay).

TABLE I

ANALYSIS OF NUCLEOTIDES OF MYOFIBRILS BY ION EXCHANGE CHROMATOGRAPHY

	μmoles/g of myofibrils			*Total*	*ADP + ATP*	$\frac{ADP}{Total}$
	AMP	*ADP*	*ATP*			
Natural myofibrils	0.87*	2.02	0.42		2.44	
Natural myofibrils	0.14	2.26	0.30	2.70	2.56	0.84
Natural myofibrils (Perry)	0.88	2.7	0.47		3.17	
Myofibrils treated by						
0.7% deoxycholate	0.14	1.74	0.14	2.02	1.88	0.86
0.7% deoxycholate	0	1.64	0.34	1.98	1.98	0.83
0.7% deoxycholate	0.07	1.83	0.17	2.07	2.00	0.88

* Probably contaminated since ratio of absorbance at 250 mμ to 260 mμ was 1.37.

Accessibility of the creatine enzyme system to myofibrils

Cr-enzyme is specific for ADP and Cr-P[17]. Hence in a suitable mixture of myofibril, Cr-enzyme and Cr-P the liberation of creatine strongly suggests that the enzyme catalyzes the reaction between ADP bound to myofibrils and the added Cr-P. In agreement with the report[5] of PERRY, we have found that in a reaction mixture of untreated myofibrils, crystallized creatine enzyme, Mg^{++} and Cr-P there was only a slight liberation of creatine.

Myofibrils treated with deoxycholate, digitonin or ATP, or myofibrils isolated from glycerinated muscle fibers catalyzed the liberation of free creatine as shown in Figs. 1 and 2. No creatine liberation was observed even with these treated myofibrils if Cr-P, Cr-enzyme, or myofibril was omitted from the reaction mixture. The slow reaction observed in the absence of added Mg^{++} may be due to Mg^{++} bound to myofibrils[18]. An explanation for the effect of treating myofibrils with ATP is not

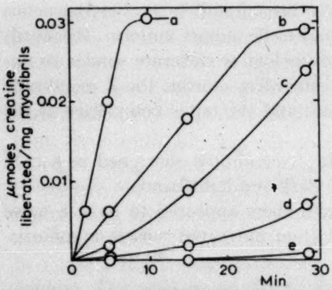

Fig. 1. Creatine liberating activity of myofibrils treated by different conditions. Creatine enzyme, 2.8 mg/ml. Creatine phosphate, 0.2 m*M*. a, myofibrils treated by 0.7% deoxycholate at pH 7.8, $\mu = 0.03$; b, myofibrils treated by 1.9% deoxycholate at pH 9.7, $\mu = 0.03$; c, myofibrils treated by 5 m*M* ATP at pH 7.0, $\mu = 0.02$; d, myofibrils treated by 1.5% digitonin at pH 7.0, $\mu = 0.47$; e, control.

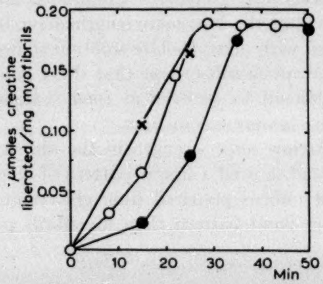

Fig. 2. Creatine liberating activity of myofibrils isolated from glycerinated psoas fibers. Creatine phosphate, 0.6 m*M*. ○, myofibrils isolated from glycerinated muscle fibers, 3.1 mg/ml. Creatine enzyme, 2.4 mg/ml. ●, same myofibrils, but 4 days after the preparation, 3.1 mg/ml. Creatine enzyme, 2.9 mg/ml. ×, same myofibrils as in ○. Myofibrils, 3.1 mg/ml. Creatine enzyme, 5.6 mg/ml.

apparent. In the case of myofibrils isolated from glycerinated fibers, the liberation of creatine showed a lag phase (Fig. 2). The lag phase was increased by aging of the myofibrils and decreased by increasing the ratio of the amount of creatine enzyme to myofibrils.

During the liberation of creatine, a gradual shortening of the myofibrils isolated from glycerinated fibers has been microscopically observed. Shortening occurs first in the H band and finally resulted in the formation of so-called contraction bands. The process seems to be very similar to the observation of HUXLEY[19] with glycerinated fibers.

Properties of creatine liberation by the myofibril-creatine enzymic system

As shown in Fig. 3, with increasing deoxycholate concentration in the washing of myofibrils, the creatine liberating activity increased to about 1% deoxycholate and then decreased when the concentration of deoxycholate was greater than 2%. There was variability in creatine liberating activity from preparation to preparation, especially when myofibrils were washed with deoxycholate concentrations greater than 0.5%.

The Mg^{++}-activated ATPase activity of myofibrils showed a slight decrease with increasing concentration of deoxycholate up to about 1% and at higher deoxycholate concentration the loss in ATPase activity was considerable (Fig. 3). There appears to be a relationship between creatine liberating activity and ATPase activity of myofibrils.

During deoxycholate treatment, large amounts of protein were extracted, as shown in Fig. 4. The nature of the protein extracted by deoxycholate has not been determined.

The creatine liberating activity of myofibrils washed by deoxycholate was not stable in borate–KCl solution stored at pH 7 and 0°. As shown in Fig. 5, stored myofibrils showed a lag phase not apparent with freshly treated myofibrils, but after overcoming the lag phase the rate of creatine liberation attained normal values. The lag phase observed with myofibrils prepared from glycerinated muscle fibers has already been pointed out (Fig. 2).

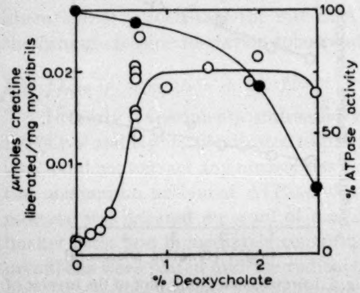

Fig. 3. Creatine liberating activity and Mg-ATPase activity of myofibrils treated by variable concentration of deoxycholate for 1 min, 0°. ○, creatine liberation. Creatine enzyme, 2.8 mg/ml. Creatine phosphate, 0.2 m*M*. ●, Mg-ATPase.

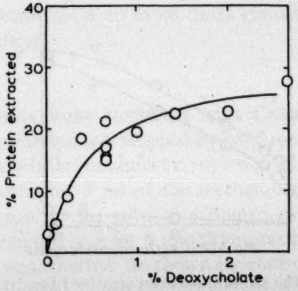

Fig. 4. Protein extraction by deoxycholate from natural myofibrils, pH 7.8, $\mu = 0.03 \pm 0.005$.

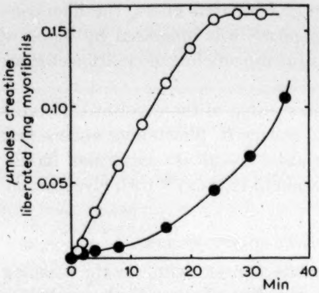

Fig. 5. Creatine liberating activities of myofibrils freshly treated by 0.7 % deoxycholate and the myofibrils stored overnight at 0°. ○, myofibrils freshly treated, 4.1 mg/ml. Creatine enzyme, 3.1 mg/ml. Creatine phosphate, 0.65 m*M*. ●, myofibrils stored, 3.9 mg/ml. Creatine enzyme, 3.1 mg/ml. Creatine phosphate, 0.7 m*M*.

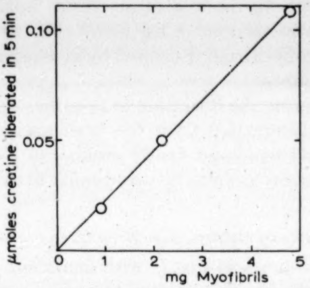

Fig. 6. The linear relationship between the activity of creatine liberation and the amount of myofibrils treated by 0.7 % deoxycholate. Creatine enzyme, 2.8 mg/ml. Creatine phosphate, 0.2 m*M*.

Fig. 6 shows the linear relationship between creatine liberating activity and the amount of myofibrils (freshly washed with deoxycholate).

Under our conditions the creatine liberating activity of myofibrils was not saturated with Cr-enzyme as shown in Fig. 7. For routine runs, Cr-enzyme was mixed with the reaction mixture shortly before the start of the reaction. When myofibrils were preincubated with Cr-enzyme in borate–KCl at pH 7 and at 0°, creatine was liberated only when further quantities of Cr-enzyme were added.

Fig. 8 shows the LINEWEAVER-BURK plots of creatine liberation. Maximum activity was reached at Cr-P concentrations greater than 1 m*M* and apparent K_m values for Cr-P of $1.05 \cdot 10^{-4}$ and $1.10 \cdot 10^{-4}$ *M* have been obtained from curves a and b respectively. However, the velocity of creatine liberation was directly proportional

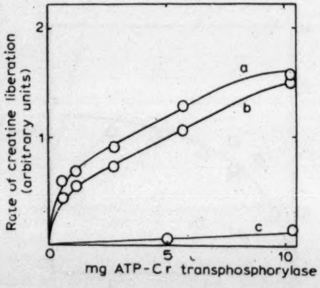

Fig. 7. The relation between creatine liberating activity and the amount of creatine enzyme on the myofibrils treated by 0.7 % deoxycholate. Creatine phosphate 0.2 m*M*. a, myofibrils treated by 0.7 % deoxycholate, 2.5 mg/ml. b, myofibrils treated by 2 % deoxycholate, 2.5 mg/ml. c, control with natural myofibrils, 3.2 mg/ml.

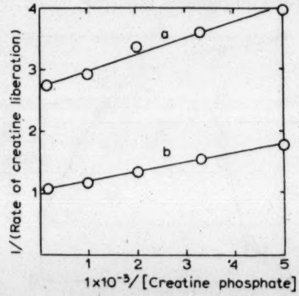

Fig. 8. LINEWEAVER-BURK plot of the inverse of the velocities of creatine liberation against the inverse of the concentrations of creatine phosphate. Myofibrils treated by 0.7 % deoxycholate. a, creatine enzyme, 2.4 mg/ml. b, creatine enzyme, 4.9 mg/ml.

to the amount of Cr-enzyme. The results suggest that the rate determining step involves the reaction catalyzed by Cr-enzyme.

Behavior of ADP on myofibrils

The myofibrils were first incubated with the complete system (myofibrils, Cr-enzyme, Mg^{++}) at pH 7 for 20 min at 30° and then were washed by borate–KCl solution. The ADP of the myofibrils was still available for creatine liberation as shown by the creatine liberating activity of a second incubation which showed the same value as the first incubation (Table II, Expts. 1 and 4, column 8). Cr-enzyme once added in the first incubation was unavailable or lost for the second incubation, but ADP was still on the myofibrils and it functioned with the same efficiency as before. These results strongly suggest that the ADP is always situated in a convenient position both for phosphorylation by the Cr-enzyme Cr-P system and for dephosphorylation by ATPase.

TABLE II

THE EFFECT OF PRE-INCUBATION ON CREATINE LIBERATING ACTIVITY OF DEOXYCHOLATE TREATED MYOFIBRILS

	Reaction mixture of the first incubation						*Creatine liberating activity of the second incubations (μmoles creatine/mg myofibrils/5 min, 30°)*	
Expt.	*Myofibrils treated by 0.7% deoxycholate (2.4 mg/ml)*	*Creatine enzyme (2.5 mg/ml)*	*$MgCl_2$ (1 mM)*	*Creatine phosphate (1 mM)*	*Temperature*	*Incubation time (min)*	*Complete system*	*Creatine enzyme is omitted from complete system*
1	+	+	+	+	30	20	0.054	0.003
2	+	—	+	—	30	20	0.048	$<$ 0.001
3	+	—	+	—	0	20	0.052	None
4	+	—	—	—	0	—	0.055	None

In Table III are given some representative data from which the figures have been drawn for the comparison of the rate of ATP hydrolysis to the rate of creatine liberation. It is seen that the rate of ATP hydrolysis is 10 to 50 times greater than the rate of creatine liberation (phosphate transfer).

Phosphate incorporation in myofibrils

In early experiments untreated myofibrils were incubated with Cr-enzyme, [^{32}P]Cr-P and Mg^{++}, all adjusted to pH 7. The reaction was stopped by 50% methanol which did not extract any nucleotides from myofibrils but almost completely inhibited the magnesium activated ATPase of the myofibrils. 1 ml of the methanol treated mixture was layered on 3 ml of 0.25 *M* sucrose in the tube of a Spinco swinging bucket rotor and immediately centrifuged at 20,000 × *g* for 3 min. The precipitated myofibrils were plated and the radioactivity was counted. As shown by control runs without creatine enzyme, the myofibrils were washed almost completely free of radioactive phosphate during the sedimentation through the sucrose layer. An excess of about $5 \cdot 10^{-4}$ μmoles of radioactive phosphate/g of myofibrils was observed when the reaction was stopped at 10 sec or 15 min. The results confirmed the previous observation of PERRY.

TABLE III

COMPARISON OF Mg-ATPASE AND CREATINE LIBERATING ACTIVITY OF MYOFIBRILS AT pH 7 AND 30°

Preparation	*Deoxycholate* %	*ATP-ase** *µmoles P/mg/min*	*Creatine liberating activity*			
			Myofibrils (mg)	*Cr-enzyme (mg)*	*CrP mM*	*µmoles Cr**/mg/min*
7	0	0.34	5.5	2.8	2.2	$<$ 0.0002
	0.02	0.35	5.4	2.8	0.2	0.0003
	0.13	0.35	5.5	2.8	0.2	0.0004
	0.26	0.31	5.1	2.8	0.2	0.0006
	0.39	0.36	5.1	2.8	0.2	0.0012
	0.70	0.33	5.0	2.8	0.2	0.003
	1.4	0.26	4.9	2.8	0.2	0.004
	2.0	0.27	4.7	2.8	0.2	0.004
	0.7		2.5	5.7	0.2	0.006
	0.7		2.5	11.4	0.2	0.007
8	0.7		2.65	2.4	5.0	0.013
	0.7		2.65	4.9	0.2	0.019
	0.7		2.65	4.9	1.0	0.019
	0.7		2.65	4.9	5.0	0.031

* Measured in the presence of 1 m*M* ATP and 5 m*M* $MgCl_2$.
** Measured in the presence of 1 m*M* $MgCl_2$.

In other experiments with myofibrils freshly washed with deoxycholate, the reaction mixtures (incubated in centrifuge tubes) were rapidly cooled in an ice–water bath, after the appropriate reaction time, and then rapidly centrifuged at 0°. The supernatant was sucked out and discarded. The bound nucleotides were extracted from sedimented myofibrils by the addition of cold TCA and were adsorbed on a column of 15 mg of Norite A mixed with cellulose powder (previously washed by acid, alkali and ethanol). The time lapse from the start of the reaction to addition of TCA was generally 20 to 25 min. However, even if *p*-CMB was added to 0.5 m*M* to stop the reaction or EDTA was added to 2 m*M* to overcome the ATPase activity by sequestering Mg^{++}[21] the amount of nucleotide containing phosphate adsorbed on Norite A did not significantly vary from $5 \cdot 10^{-3}$ µmoles/g of myofibrils.

In later experiments on phosphate incorporation by deoxycholate treated myofibrils, the procedure used was that described above. The curves indicating the amount of radioactive nucleotide incorporated against the reaction time were obtained as shown in Figs. 9 and 10. The incorporation rises to a maximum in 1 to 2 min and then shows a decline. The amount of radioactive nucleotide increased with increase in concentration of [^{32}P]Cr-P added (Fig. 10). The maximum incorporation of ^{32}P from [^{32}P]Cr-P that we have observed (conditions may not yet be ideal) has been 40% of the ADP and ATP bound to the myofibrils treated with deoxycholate. Untreated myofibrils or control runs in which the TCA was added before the substrate showed essentially no incorporation of the phosphorus isotope.

Identification of radioactive nucleotide

Ion exchange chromatography (Dowex-1) of nucleotides[22] extracted from myofibrils incubated with the complete system showed three peaks of AMP, ADP and ATP by u.v. absorption and the ATP peak contained essentially all of the radioactivity, excluding ^{32}Pi and [^{32}P]Cr-P (Fig. 11).

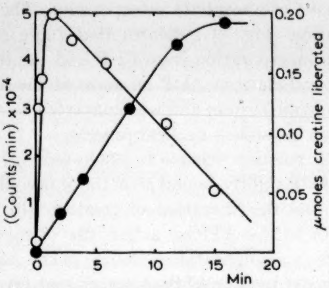

Fig. 9. Radioactive phosphate incorporation in the myofibrils treated by 0.7 % deoxycholate with creatine enzyme system, and the creatine liberating activity with the same myofibrils and the enzyme system. ○, phosphate incorporation. Radioactive creatine phosphate, 0.2 m*M* and $2.3 \cdot 10^7$ counts/min. Myofibrils, 19.5 mg/5 ml. Creatine enzyme, 15.4 mg/5 ml. ●, creatine liberating activity. Creatine phosphate, 0.2 m*M*. Myofibrils, 3.9 mg/ml. Creatine enzyme, 3 mg/ml.

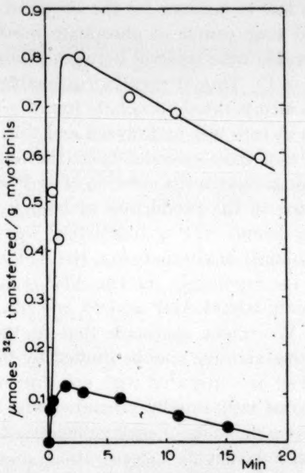

Fig. 10. Radioactive phosphate incorporation in the myofibrils treated by 0.7 % deoxycholate with creatine enzyme system at different creatine phosphate concentrations. ○, radioactive creatine phosphate, 0.75 m*M* and $1 \cdot 10^7$ counts/min. Myofibrils, 19.4 mg/5 ml. Creatine enzyme, 15.4 mg/5 ml. ●, radioactive creatine phosphate 0.2 m*M*. Myofibrils, 19.5 mg/5 ml. Creatine enzyme, 15.4 mg/5 ml.

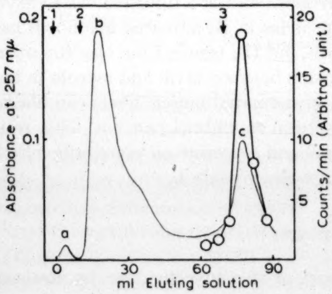

Fig. 11. Ion exchange chromatography of nucleotides extracted from myofibrils treated by 0.7 % deoxycholate. Radioactive creatine phosphate, 0.16 m*M* and $9.5 \cdot 10^6$ counts/min. Myofibrils, about 130 mg/15.5 ml. Creatine enzyme, 24.3 mg/15.5 ml. Eluent at 1 is 0.2 *M* formic acid–0.3 *M* ammonium formate; at 2, 2.5 *M* formic acid–0.3 *M* ammonium formate; and at 3, 4 *M* formic acid–0.3 *M* ammonium formate. Peak a, AMP; b, ADP; c, ATP. ○, radioactivity.

DISCUSSION

The reaction system containing Cr-enzyme and deoxycholate-washed myofibrils, liberated creatine and produced bound ATP without addition of ADP, *i.e.*, ADP bound to myofibrils was phosphorylated by the Cr-enzyme system and the nucleotide remained bound to the myofibril. Subsequently the bound ATP was hydrolyzed by the Mg-ATPase of myofibrils to bound ADP and inorganic phosphate so that the net reaction is the breakdown of Cr-P. The same Mg-ATPase activity of myofibrils was maintained during the reaction cycle. The highest phosphate incorporation observed by us was 40 % of the total amount of ADP plus ATP bound to myofibrils.

As shown in Table I, more than 80 % of the nucleotides of myofibrils was ADP. It is generally assumed that the nucleotides of myofibrils are bound to actin. It should be noted that preparations of myosin also contain ADP and ATP[23], but the amount is too low to account for the observed amount of phosphate incorporation. The observed time course of phosphate incorporation (Fig. 9) indicates that there is an observable time interval between phosphate incorporation from Cr-P and the liberation of Cr. Thus it may be reasonable to conclude that ADP on actin of the myofibrils incorporated phosphate from the Cr-enzyme system and was converted to ATP which in turn was hydrolyzed and thus makes possible a cyclical process.

From these considerations the following reaction scheme is suggested: (a) Cr-enzyme catalyzes the reaction of Cr-P with ADP tightly bound to actin of myofibrils resulting in the production of bound ATP and the liberation of creatine. (b) The loosely bound ATP is transferred from actin to the ATPase active site of myosin in myofibrils or alternatively, the ATP induces a configurational change in the make-up of the myofibrils. (c) The ATP is hydrolyzed by the ATPase activity of myosin to loosely bound ADP and Pi and (d) the ADP formed is returned to the original state. We might speculate that in this system under consideration the creatine liberating activity may be limited by the degree to which the nucleotide sites of the myofibril are saturated with creatine enzyme, since (a) the myofibril system seems to become very rapidly saturated with regard to formation of bound ATP; and (b) increasing amounts of enzyme increased creatine liberating activity (Fig. 8) although even at low enzyme concentrations many-fold excess enzyme (on the basis of assay in a purified system) had been added for the observed activity.

The accessibility of myofibril for the Cr-enzyme system might depend on a steric interrelation of actin and myosin inside the myofibril and/or a diffusion of Cr-enzyme into the myofibrils. There is no evidence that proteins in glycerinated myofibrils have the same configuration as in the intact myofibrils, but the results from electron microscopy suggest the same structural interrelationship between actin and myosin in both kinds of myofibrils. However, myofibrils from glycerinated muscle fibers can liberate creatine from the Cr-enzyme system while natural myofibrils can not. This result seems to suggest that the effect of deoxycholate and glycerine on myofibrils may be on the penetration of the Cr-enzyme system into the myofibril.

ACKNOWLEDGEMENT

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A RE-EXAMINATION OF THE MOLECULAR CHARACTERISTICS OF G-ACTIN

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SUMMARY

1. The molecular weight of ultracentrifugally homogeneous G-actin, prepared by the method of Tsao and Bailey, is 66,000 ± 2,000 by light scattering and 61,600 ± 6,000 by sedimentation viscosity.

2. The virtual identity of the extrapolated Hc/τ intercept found by light scattering for G-actin in either 0.5 M KI or H_2O-10^{-4} M ATP suggests that the same limit of dissociation is attained for the protein dissolved in either of these media.

3. The light scattering data do not support the earlier view, derived from osmotic pressure measurements, that both monomeric and dimeric G-actin exist in depolymerized actin systems.

4. The dispersion constant, $\lambda_0 = 223$ mμ, is unusually low for G-actin, reflecting an apparent lack of any extensive helical configuration in the molecule.

INTRODUCTION

The earliest attempts to characterize monomeric G-actin were limited by the lack of purity of the preparations used[1–3]. Most of these studies were carried out on preparations based on the original or modified procedures of Straub[4], and these were subsequently shown to contain as much as 40–60 % impurity[5,6]. In 1951 Mommaerts[5], using the method of successive polymerizations and ultracentrifugal separation of the polymerized actin, was able to obtain preparations of G-actin that showed a single broad peak in the ultracentrifuge, with an intrinsic sedimentation constant of 2.8 S when examined in the stabilizing medium of 10^{-4} M ATP, pH 8–8.2. The molecular weight as calculated from measurements of 90° scattering in this same medium was 57,000 (see ref. 1) as compared with a value of 80,000 obtained by STEINER, LAKI AND SPICER[3], also by light scattering, on unpurified preparations in 0.5 M KI, a medium known to favor the depolymerization of polymeric F-actin to G-actin. This discrepancy was no doubt due very largely to the difference in purity of the preparations, but what contribution the different media may have made to it remained an unanswered question.

In 1953 TSAO AND BAILEY[8] evolved their own variation of the STRAUB procedure to obtain electrophoretically homogeneous G-actin. In this work, the protein samples were not purified by fractional sedimentation in the ultracentrifuge, but, rather, the final extraction was made with 30 % acetone instead of pure water, a treatment which successfully suppresses the extraction of contaminants, notably tropomyosin. Various preparations were subjected to osmotic pressure measurements in 0.6 M KI (see ref. 9). The results were found to be somewhat erratic, indicating in some experiments the presence of a 74,000 unit, at other times one of 140,000, and in very dilute solution the transition of the latter to the former with progressive dilution. Viscosity determinations on a G-actin preparation, which by osmotic pressure measurements possessed a particle weight of 74,000, gave an intrinsic viscosity value of 0.21 dl/g, indicating, for a prolate ellipsoid of revolution, an axial ratio of about 12. The use of an assumed $\bar{v}$ value for actin of 0.74 in conjunction with this viscosity provided dimensions of 290 Å and 24 Å for the length and diameter of the monomer.

More recently TSAO's[9] work has come under severe criticism from GEIDUSCHEK AND HOLTZER[10], who point out that the osmotic pressure data must be discounted, because the extrapolation is not unique for the various samples examined, even though they were gathered under presumably identical conditions. The viscosity results are also questioned in view of their lack of parallellism with the osmotic pressure measurements. These reviewers argue that, since the osmotic pressure data on the identical sample in the same medium show the occurrence of distinct dissociation upon dilution of the protein, the viscosity curve should also show strong negative curvature in this region. The fact that it does not indicates that the two sets of measurements are inconsistent.

From the foregoing discussion it is apparent that although considerable progress has been made in elucidating the actin system, several "sore-spots" in the story must be re-explored. Among these is the question of the disparity in the fundamental particle weight of G-actin in the water–ATP system and in 0.5 M KI: to date none of the better preparations have been subjected to molecular weight determination by at least two different methods. Secondly, the evidence of TSAO for the existence of monomeric and dimeric G-actin in 0.6 M KI is far from conclusive and deserves re-investigating. For this purpose light scattering is the preferred method over osmotic pressure because of its accessibility to lower protein concentrations with a resultant smaller uncertainty associated in the extrapolated ordinate intercepts. The present investigation was carried out with a view to answering the above questions. For this purpose, a preparation of G-actin prepared by the method of TSAO AND BAILEY was studied, using sedimentation, viscosity, light scattering and pycnometric techniques.

Also, an estimate of the helical content of the protein was obtained through a study of its optical rotatory dispersion.

MATERIALS AND METHODS

Preparation of G-actin

The protein was prepared from rabbit skeletal muscle by the method of TSAO AND BAILEY[8]. Material prepared in this way was shown by the above workers to be electrophoretically homogeneous at pH 2 and 10. However, since electrophoresis patterns do not show up the presence of stepwise aggregates of the same molecule, ultracentrifugation was chosen as the criterion of purity in this study. An example of the ultracentrifugal pattern obtained for G-actin in water–10^{-4} M ATP at pH 8 is given in Fig. 1.

A single broad asymmetric peak, similar to that obtained by MOMMAERTS[5] for material prepared by his method, was observed. Although this is not regarded as a rigorous indication of purity, it must be stressed that there is a special difficulty in the case of G-actin: it is stable only at low ionic strength and mildly alkaline pH in the presence of ATP. MOMMAERTS has shown that under these conditions charge effects and the sedimentation of ATP itself complicate the interpretation of the boundary[7]. Furthermore, since ordinary preparations exhibit more than one peak under similar conditions, it may be inferred that the purification procedure has been at least partially successful. For most of the studies reported herein, solutions of G-actin in water–10^{-4} M ATP were dialyzed against repeated changes of KI solution (0.5 M KI, 0.05 M K_2HPO_4, 0.025 M KH_2PO_4, pH 7, ionic strength = 0.6). Representative photographs of a sedimentation–velocity run on such a preparation is shown in Fig. 2, from which it is seen that a single boundary was maintained throughout the course of the run.

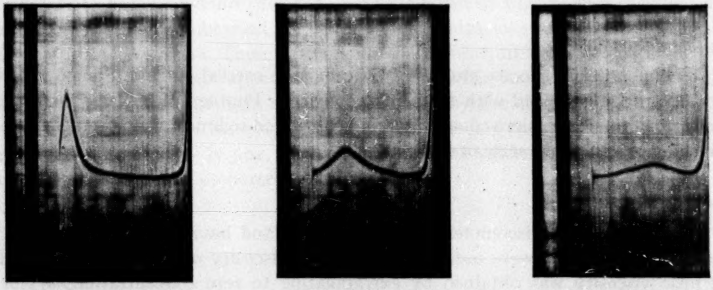

Fig. 1. Ultracentrifugal sedimentation patterns of 0.56 % G-actin in H_2O–ATP (10^{-4} M) at pH 8. Centrifugation at 59,780 rev./min in Kel-F cell; bar angle, 50°; temperature 23.5°; time after reaching full speed: a, 16 min; b, 80 min; c, 144 min.

Protein concentrations were determined by the micro-Kjeldahl method, taking 16.1 % for the total nitrogen[9]. Concentrations in the ultracentrifuge runs were usually checked by area measurements of tracings of enlarged patterns (10 ×) made with a photographic enlarger. The concentration of sedimenting material was calculated in accordance with the equation given by SCHACHMAN[11].

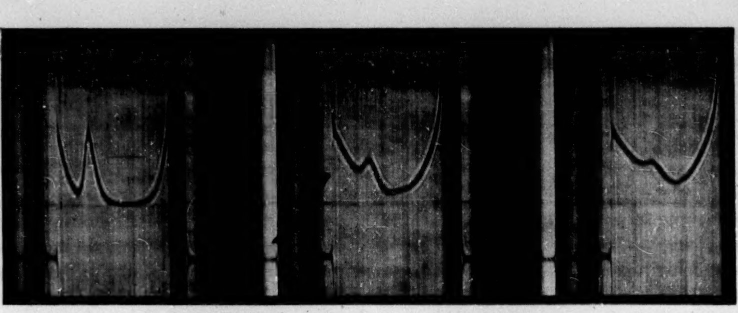

Fig. 2. Representative photographs obtained in a sedimentation–velocity run for 0.34 % G-actin in 0.5 M KI, I = 0.1, phosphate buffer, pH 7. Centrifugation at 59,780 rev./min in synthetic boundary cell; bar angle, 60°; temperature, 24.5°; time after reaching full speed: a, 0 time; b, 16 min; c, 32 min. Much of the curvature of the pattern on the left is due to the redistribution of the KI buffer solution.

Sedimentation

Sedimentation velocity studies of G-actin were carried out in a Spinco model E ultracentrifuge equipped with temperature control. Photographic plates were measured with a Gaertner micro comparator and calculated sedimentation coefficients were corrected to a standard state of water at 20°.

Intrinsic viscosity

Ostwald-Fenske viscometers requiring 6.5 ml and having a water flow time of about 240 sec at 25° were used to measure the viscosity of G-actin solutions. The intrinsic viscosity was obtained by extrapolating to zero concentration, a plot of reduced specific viscosity, η_{sp}/c, against c in g/100 ml.

Light scattering

Light scattering measurements were performed at a wavelength of 436 mμ with the Brice-Phoenix light scattering photometer. Prior to the measurements the protein solutions were clarified by filtration through millipore filters as described by KAY AND EDSALL[13]. Turbidities on the pure solvents were determined in a similar fashion and were subtracted from the readings obtained with actin solutions. Scattering dissymmetries at 45° and 135° and depolarization measurements were carried out in the Brice instrument in the prescribed manner. The specific refractive index increment of G-actin, determined in a Phoenix differential refractometer[14], was 0.172 at 4358 Å and 25°, in good agreement with the previously published figure[7].

Partial specific volume

Partial specific volume determinations were made at 20 ± 0.01° with pycnometers of 10 ml capacity, which were calibrated with air-free distilled water. Partial specific volumes were calculated from the equation[15]:

$$1 - \bar{v}\rho = \frac{1 - W_2}{m} \cdot \frac{\mathrm{d}m}{\mathrm{d}W_2} \qquad (1)$$

where m is the mass of solution, W_2 is the weight fraction of the protein (0.01 times the concentration by weight) and ρ is the density of the solution.

*Optical rotary dispersion measurements**

Optical rotations were measured with a Rudolph automatic recording spectropolarimeter, Model 260. The light source was a Xenon compact arc lamp and the wavelength range investigated extended from 300 to 650 mμ. The concentration of the protein solution, contained in 1-dm tubes, was 0.3 % and the temperature of the measurement was 29°.

RESULTS AND DISCUSSION

Sedimentation

A plot of the calculated $S_{20,w}$ values against concentration for G-actin in the KI–phosphate buffer medium (pH 7, I = 0.6) is given in Fig. 3. Also included are 3 sedimentation values obtained for different concentrations of the protein in the H_2O–10^{-4} M ATP system. These latter values are susceptible to serious error due to charge effects, but do not appear to be out of line with the values obtained in the presence of salt. All of the data can be represented by the single full line of Fig. 3 derived by the method of least squares. The sedimentation constant extrapolated to zero concentration ($S_{20,w}$) is 3.02, as compared with the values of 2.8–3.1 obtained by MOMMAERTS for material prepared by his method.

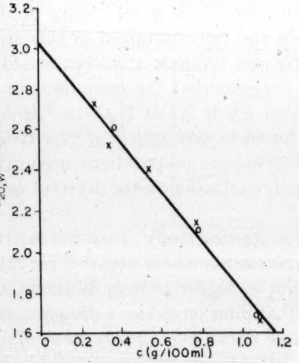

Fig. 3. Concentration dependence of sedimentation of G-actin prepared by the method of TSAO AND BAILEY[8]. The crosses represent a solvent medium of 0.5 M KI, 0.1 M K phosphate (pH 7, I = 0.6) and the circles a medium of H_2O–10^{-4} M ATP at pH 8.

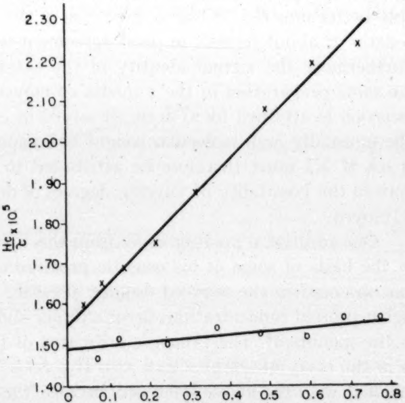

Fig. 4 Hc/τ *versus* c data for G-actin in H_2O–10^{-4} M ATP (×) and in phosphate buffered KI of pH 7 and ionic strength 0.6(○).

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* These were kindly performed by M. M. MARSH of the Lilly Research Laboratories, Indianapolis, Ind. (U.S.A.).

Light scattering

The values of Hc/τ as obtained for G-actin in the solvent media explored are plotted as a function of c in Fig. 4, where the best straight lines have been drawn by the method of least squares.

Turbidity measurements made at 45° and 135° to the incident beam indicated that no dissymmetry corrections were necessary and that the protein molecules were smaller in size than 0.1 × the wavelength of the light employed, which is approximately 3250 Å (4358/1.34). Depolarization was also negligible; hence no depolarization correction was required. The intercept of Hc/τ at zero concentration is the reciprocal of the molecular weight, M, according to the equation:

$$\frac{Hc}{\tau} = \frac{1}{\mathrm{M}} + 2Bc \tag{2}$$

It is to be noted that in the water–ATP medium at pH 8 the second virial coefficient, B, has a high positive value, reflecting the repulsive forces between the charged protein molecules. On the other hand, as is usual in protein solutions at high ionic strengths, there is little concentration dependence of Hc/τ in the phosphate-buffered KI solvent (pH 7, ionic strength 0.6). The observed ordinate intercept values of $1.55\ (\pm\ 0.04)\cdot 10^{-5}$ and $1.49\ (\pm\ 0.05)\cdot 10^{-5}$ for G-actin in the water–ATP medium and in the KI buffer solution respectively are within experimental error; for this reason an average figure of $1.52\ (\pm\ 0.04)\cdot 10^{-5}$ was chosen, corresponding to a molecular weight of 66,000 ± 2000. This is considerably higher than the value of 57,000 obtained by MOMMAERTS for material prepared by his technique[7]. However, the latter figure includes a depolarization correction which, in view of GEIDUSCHEK's work[16], is probably better omitted. If this is done, use of the intercept alone gives a value of M for G-actin of about 62,000, in good agreement with the value obtained in this study. Furthermore the virtual identity of the extrapolated ordinate intercept found for the same preparation in the 2 media employed suggests that the same limit of dissociation is attained for G-actin dissolved in either 0.5 M KI or H_2O–10^{-4} M ATP. The unusually high molecular weight of 80,000 found by STEINER *et al.*[3] for G-actin in 0.5 M KI must therefore be attributed to the impure preparations used rather than to the possibility of varying degrees of depolymerization in the different media employed.

One additional point emerges from this light scattering study: TSAO has inferred, on the basis of some of his osmotic pressure experiments where negative curvature was observed in the reduced osmotic pressure plot at higher protein dilutions, that higher protein concentrations favor a dimer and that dilution causes a disaggregation to the monomer[9]. The principle criticisms of these data have already been alluded to in the essay of GEIDUSCHEK AND HOLTZER[10]. In addition, the present study is at variance with the above inference, because the light scattering curves in both media do not exhibit any upsweep in the values of Hc/τ upon dilution. Since these measurements were extended to a protein concentration some 10 times lower than the osmotic pressure work, their greater reliability is unquestioned.

Viscosity

Results of viscosity measurements on a G-actin preparation in 0.5 M KI–0.1 M phosphate at pH 7 are shown in Fig. 5.

As in most proteins at high ionic strength, there is little concentration dependence of η_{sp}/c. The intrinsic viscosity obtained, 0.14 dl/g, is at variance with the value of 0.21 reported by TSAO for G-actin in 0.6 M KI + 10^{-4} M ATP. However, the latter figure has been questioned in view of the inconsistencies observed between TSAO's osmotic pressure and viscosity measurements[10]. Moreover, since in this study good reproducibility was obtained in the viscosity determinations made at each dilution, the figure of 0.14 dl/g will be considered the more reliable and will be used in all calculations involving $[\eta]$.

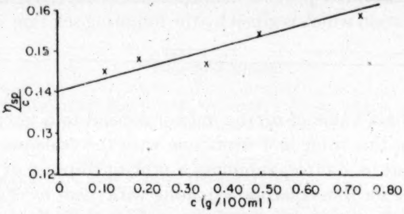

Fig. 5. Reduced specific viscosity of G-actin in phosphate-buffered KI (pH 7, ionic strength 0.6) as a function of protein concentration.

Partial specific volume

Fig. 6 shows a plot of m, the mass of solution held by the pycnometer to w_2, the weight fraction of solute for G-actin in phosphate-buffered KI (pH 7, ionic strength 0.6).

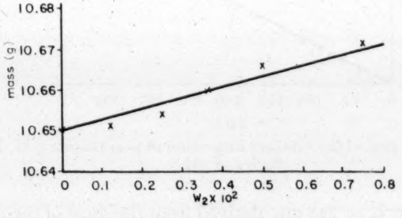

Fig. 6. Plot of m (mass of a given volume of solution of G-actin for which the solute weight fraction is w_2) against w_2.

The slope of the line, dm/dw_2, was drawn according to the method of least squares and when used in equation (1) gave a value of 0.716 ± 0.005 for the partial specific volume.

Molecular weight from sedimentation–viscosity

Another estimate of the molecular weight can be obtained from sedimentation and viscosity data by use of the SCHERAGA-MANDELKERN equation[17]:

$$\mathrm{M} = \left[\frac{Ns[\eta]^{1/3}\eta_0}{\beta(1-\bar{v}\rho)}\right]^{3/2} \tag{3}$$

In this equation N is Avogadro's number, s is the measured sedimentation coefficient extrapolated to infinite dilution of the protein, $[\eta]$ the weight intrinsic viscosity, η_0 the viscosity of the solvent, $\bar{v}$ the partial specific volume of the protein in the solvent and ρ the solvent density. The parameter β depends on the hydrodynamic characteristics of the solvated protein molecule and is derivable from the viscosity increment, γ, of the protein which is given by the following relation:

$$\gamma = \frac{[\eta]\cdot 100}{\bar{v}} \tag{4}$$

A $[\eta]$ of 0.14 dl/g and a $\bar{v}$ value of 0.716 g/ml correspond to a viscosity increment of 19.5 for G-actin. Using this value in conjunction with the table provided by SCHACHMAN[18] leads to a β value of $2.5 \cdot 10^6$, assuming a prolate ellipsoid of revolution model, Substituting this value for β in equation (3) along with $3.02 \cdot 10^{-13}$ sec for s, 0.14 dl/g for $[\eta]$, 0.00975 poise for η_0, 0.716 ml/g for $\bar{v}$, 1.065 g/ml for ρ and solving for M, leads to a value of 61,600 ± 6000, in good agreement with the 66,000 ± 2000 value deduced by light scattering.

Optical rotation

The optical rotatory dispersion for G-actin in 0.5 M KI–0.1 M phosphate buffer of pH 7, shown in Fig. 7, was plotted using the simple one-term DRUDE equation: $[\alpha]_\lambda = k/(\lambda^2 - \lambda_0^2)$ and modified plot suggested by YANG AND DOTY[19]. Although the specific rotation at 550 mμ is within the range of globular proteins, *i.e.* $[\alpha]_{550} = -60°$, the dispersion constant, $\lambda_0 = 223$ mμ, derived from the slope of the plot of $\lambda^2[\alpha]$ *versus* $[\alpha]$, is unusually low, reflecting an apparent lack of any extensive helical configuration in the molecule. The percent helix was estimated from the λ_0 value, in accordance with the method described by YANG AND DOTY, as 8 %. This low helix value is no doubt correlated with the high proline content in the molecule (29 residues/molecular weight of 65,000 (see ref. 20)). If these were distributed statistically along the polypeptide chain, only short helical segments could exist, since the presence of a prolyl residue in the chain interrupts the continuity of the helix.

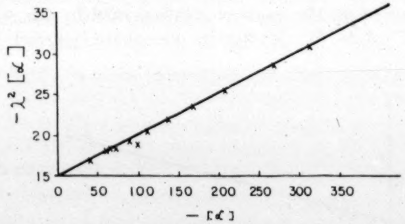

Fig. 7. A modified DRUDE plot of the rotatory dispersion of G-actin in 0.5 M, KI–0.1 M phosphate buffer of pH 7.

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THE UPTAKE OF AMINO ACIDS BY ISOLATED SEGMENTS OF RAT INTESTINE

I. A SURVEY OF FACTORS AFFECTING THE MEASUREMENT OF UPTAKE

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SUMMARY

Techniques have been developed for the determination of the uptake of a large number of amino acids on a single preparation of segments from isolated small intestine of rats. The uptake can be followed by the fall in amino acid concentration of the external medium provided that a correction is made for water exchange between the tissue and the medium. As measured in this way the uptake may include both accumulation within, and metabolic transformation by the tissue. From recovery studies, metabolic transformation is likely to be of importance only in the uptake of L-ornithine, L-arginine, L-aspartic acid, L-glutamic acid and L-glutamine. The amino acids that are completely recoverable from the tissue after uptake show similar time-progresses of uptake with a cessation of net uptake suggestive of a steady state. The uptakes of all the amino acids, with the possible exceptions of L-arginine and L-lysine, are inhibited by 2,4-dinitrophenol. It is concluded that comparisons of rates of uptake may be made by determination of the uptake at 4 min.

INTRODUCTION

Isolated preparations of tissue from small intestine will transfer certain L-amino acids against a concentration gradient[1-6]. Similar preparations will also take up L-histidine and concentrate it within the tissue[7]. These results would indicate a transport mechanism requiring energy. Impairment of the concentrating process when the supply of metabolic energy is impaired, as in anoxia[6] and in the presence of cyanide or 2,4-dinitrophenol[4,7], is supporting evidence of an active process for transport of amino acids in intestinal tissue. In investigating the rate of such a process a number of factors may affect the result obtained. Since the range of conditions for the physiological activity of the tissue is limited, these factors cannot be eliminated, and so must be assessed rather than controlled. The purpose of the present paper is to survey the uptake of amino acids to seek information on some factors of possible effect on the determination of uptake.

EXPERIMENTAL METHODS

Preparations of tissue

Segments of intestine were prepared from starved, young female rats (120–170 g) as described by AGAR *et al.*[7]. In some experiments on water exchange the procedure of these authors was followed in detail, using rats starved 18–24 h and washing the segments by at least 5 min preincubation. Otherwise the rats were starved 40–48 h, and the tissue was washed twice in 50 ml of Krebs bicarbonate–Ringer buffer[8] (pH 7.4) containing 0.5 % glucose, gassed with CO_2–O_2 (5:95). When the tissue was taken from more than one animal, it was all mixed in the first washing which was continued for 5 min after the addition of the last lot of tissue; it was then transferred to the second vessel for a further washing of 10 min. This gave a total preincubation time of 15–21 min, the latter time being that for the tissue from the first rat. On removal from the washing vessel the segments were drained and turned over once on hardened filter paper before transfer to the incubation medium.

Conditions of incubation

The tissue was incubated in a medium of Krebs bicarbonate–Ringer buffer (pH 7.4) containing 0.5 % glucose and amino acids as required, shaken in 25-ml conical flasks, with a gas phase of CO_2–O_2 (5:95). Samples were taken before and at intervals, usually 4, 10 and 50 min, after the addition of tissue. The dry weight was determined after drying the tissue overnight at 110°. The uptake of amino acid in µmole/g dry weight was calculated from the decrease in concentration of the external solution, making allowance for the change in volume of medium resulting from the exchange of water between the tissue and the medium under the conditions of the experiment.

In experiments with amino acids at 10 mM, 0.15–0.20 g (dry wt.) of tissue was incubated in 4 or 5 ml of medium. For concentrations above 10 mM, the volume was 3 ml. At 1 mM concentration, 0.1 g (dry wt.) was incubated in 5 ml. A correction was made for the amino acid released from control tissue incubated in amino acid-free buffer. The magnitude of this correction varied with the tissue and the amino acid, ranging from 1 µmole/g dry weight (*e.g.* valine, methionine) up to 5 µmoles/g dry weight (alanine). The release of amino acids from the tissue was not greatly affected by the presence of other non-metabolised amino acids in the medium.

Investigation of water exchange

The exchange of water was followed by weighing samples of tissue after they had been carefully freed of surplus water by being turned over on hardened filter paper. The difference between the weights before (preincubation fresh weight) and after (post-incubation wet weight) the final incubation period was taken as the exchange of water by the tissue. Replications were made with each batch of tissue, the number of replications depending on the type of experiment.

Recovery of amino acid taken up

Tissue (1.0–1.2 g wet weight) prepared normally was incubated in 5 ml of the appropriate medium of buffer + 10 mM amino acid, or buffer alone for the control. After 50 min, 3 ml of the incubating medium was withdrawn and made up to 8 ml for estimation. The remaining medium was withdrawn, combined with three subsequent ethanolic extracts of the tissue and made up to 8 ml. A further four washings were made with 2 ml volumes of 70% ethanol, then combined and made up to 8 ml. 5-ml samples of the two initial media (with and without amino acid) were each made up to 8 ml. Any turbidity which appeared in the extracts on standing was removed by centrifugation.

The amount of amino acid disappearing from the external medium was determined from the concentration of the medium before and after incubation. On the samples obtained by pooling equal volumes of the appropriate solutions, the initial and final contents of amino acid in the whole system were estimated in triplicate. For the initial content, the sample was derived from the unincubated amino acid-containing medium, plus the medium and extracts from the control incubation. For the final content, the sample was derived from the unincubated amino acid-free medium, plus the medium and extracts from the incubation with amino acid. The difference between initial and final amounts of the amino acid has been taken as the loss due to the metabolism of the amino acid by the tissue. Amino acids formed as products of metabolism were detected by comparing the chromatograms. Where the formation of an amino acid appeared likely from this comparison, the initial and final amounts of it were estimated quantitatively.

Tissue extracted as above adsorbs lysine strongly and does not release it to further washing with 70% ethanol. Extraction with 1% Na_2CO_3 (w/v) in 70% ethanol yielded higher recoveries of lysine, ornithine and arginine.

Estimation of amino acids

Except for proline, and in some cases for histidine, the concentration of amino acids was measured by a paper chromatographic method[9]. 0.1–0.2 μmole of amino acid was applied to the paper and the chromatograms developed with a descending solvent, using *n*-butanol–acetic acid–water (4:1:1, v/v) for all amino acids except alanine, for which *n*-propanol–water (4:1, v/v) was used. Replicates were found to be within a range of ± 2% of the mean. For histidine, the results were similar to those from the method of MacPherson[10]. Proline was estimated by the method of Chinard[11]. Although the colour development was markedly stimulated by the presence of other amino acids, the effect did not seriously interfere with the determination of the uptake of proline.

RESULTS AND DISCUSSION

Water exchange

Preliminary experiments indicated that uptake of water occurs during the period of preincubation and that it is substantially complete within 15 min. After preincubation while the tissue is out of the aqueous medium for distribution, a release of water on to the surface of the tissue occurs. This water is conveyed with the tissue to the incubation medium, when a renewed uptake of water begins. The dilution of the incubation medium at a given time will depend on the counter-balancing effects of the initial addition of water and its removal by the renewed uptake. For a net release of W ml/g dry weight of tissue the correction to the uptake determined from a final amino acid concentration of C_2 mM would be $-WC_2$ μmole/g dry weight. To allow this correction the exchanges of water between tissue and medium were studied using the same procedures and times as in determining the uptake of amino acids.

Results are presented in Tables I and II. The release after 4 min incubation is significantly less (at the 1% level) for tissue preincubated 5 min than for tissue preincubated 10 min, while there is not a significant difference between the effects of 10 and 15 min preincubations (Table I). With tissue preincubated 15 min and incubated 4 min, the release is significantly less (at the 5% level) for tissue from rats starved 24 h than that for tissue from starved 48 h (Table I). There was no significant difference beween the variance of samples taken from a single rat and of samples taken from the pooled tissue of 4 rats. In the experiments with tissue, pooled for 15–21 min preincubation, from four rats starved 48 h, the results summarized in Table II show no significant differences at the 5% level in the effect of the pairs of incubation treatments on the net release of water. The significantly greater release for no incubation than for 4 min incubation supports the observation that water is released during the handling of the tissue and is taken up on incubation.

TABLE I

WATER EXCHANGE BY SEGMENTS OF RAT INTESTINE

Segments of small intestine, from rats starved 24 h were preincubated for the given times, then three samples from each rat were transferred for incubation for the given times. Water exchange determined as described in text.

Period of preincubation (min)	*Period of final incubation (min)*	*Number of samples*	*Average change of wt. (g/g preincubation fresh wt.)*	*Average dry wt. (% preincubation fresh wt.)*
5	4	36	—0.040	—
5	10	30	—0.026	—
5	15	30	—0.021	—
5	25	30	+0.006	—
5	50	26	+0.031*	16.9**
10	4	24	—0.075	—
15	4	39	—0.074	16.8***
15§	4§	33§	—0.087§	16.0§

* Values for one rat giving negative water exchange deleted, since not homogenous with the remainder.

** Based on values for 6 rats, 17 samples.

*** Based on values for 6 rats, 18 samples.

§ Samples from rats starved 48 h.

For corrections in this paper only the values for tissue, from rats starved 48 h, pooled for 15–21 min preincubation have been required. Since the effects of the various incubation treatments in Table II are not significantly different from the corresponding ones with L-histidine alone, all corrections have been made on the basis of the average values for the net release after incubation in the presence of 10 m*M* L-histidine. Equating grams of weight lost with millilitres of water released, these values are 0.76 ml/g dry weight for no incubation, 0.64 ml/g dry weight for 4 min incubation, 0.46 ml/g dry weight for 10 min incubation and 0.59 ml/g dry weight for 50 min incubation. For uptake at 4 min, determined at 10 m*M*, this would correspond to a correction of —6.4 μmoles/g dry weight, with an extreme range of

TABLE II

WATER EXCHANGES BY SEGMENTS OF RAT INTESTINE INCUBATED IN THE PRESENCE OF L-AMINO ACIDS

Segments of small intestine, from 4 rats starved 48 h, pooled and preincubated 21–15 min and then incubated for the given time with L-amino acids at 10 m*M* and 2,4-dinitrophenol as noted. Water exchange determined as described in text. Treatments bracketed together were performed on samples from the same preparation of tissue. The statistical significance of the differences between these treatments are given (5% = significant at 5% level; N.S. = not significant at 5% level).

Additions to buffer plus glucose	*Period of final incubation (min)*	*Number of samples*	*Average change of wt. (g/g dry wt.)*		*Average dry wt. (% preincubation fresh wt.)*
Histidine	4	12	0.65		15.2
Isoleucine	4	8	0.68		16.0
Histidine	4	4	0.69	N.S.	15.6
Isoleucine	4	4	0.61		15.5
Histidine	4	6	0.58	N.S.	16.0
Histidine + isoleucine	4	6	0.61		16.1
Histidine	4	4	0.68	N.S.	15.4
Histidine + DNP	4	4	0.63		15.0
Histidine	10	4	0.46	N.S.*	16.1
Histidine + DNP	10	4	0.64		15.1
Histidine	50	2	0.59	N.S.	14.8
Histidine + DNP	50	2	0.69		14.2
Histidine**	4	4	0.62	5%	16.4
Histidine**	Nil	4	0.76		16.7
Histidine***	50	4	0.32	N.S.	15.6
Histidine + isoleucine***	50	4	0.43		15.7

* Large difference required for significance due to one anomalous figure which cannot be rejected.

** 4 samples from the pooled tissue of 2 rats.

*** Samples of segments preincubated 10–5 min, from rats starved 24 h.

error of ±2.5 μmoles/g dry weight as shown from the extreme ranges of a total of 52 determinations including those in the presence of L-isoleucine and 2,4-dinitrophenol.

The net decrease in weight, for tissue from rats starved 48 h, over 50 min incubation following 15–21 min preincubation might suggest that the water released during the distribution procedure has not been replaced. However, it seems likely that this loss of weight may represent a loss of tissue, since the dry weight as a percentage of the preincubation fresh weight has dropped considerably (Table II). Such loss of tissue is observed to occur after long periods of incubation and may be interpreted as a shedding of the mucosa. The correction has been applied since the material lost may also have a diluting effect. For the greater uptake at 50 min than at earlier times, the relative magnitude of the correction is small.

Variability of rates of uptake

Fig. 1 shows an example of the variations in the rate of uptake of L-alanine by a single preparation of tissue. Because of the lesser variation in the uptake determined at 4 min, as compared with those determined at later times, it was decided to base estimates of rate of uptake on that at 4 min. A further advantage of measuring uptake at 4 min, as compared with later times, is that the net rate of entry will be closer to the initial rate of entry into the tissue. For the uptake at 4 min of L-alanine at 10 m*M*, two single preparations of tissue showed ranges of 38–44 μmoles/g dry weight for four replicates, and 43–50 μmoles/g dry weight for six replicates, as compared with a range of 35–56 μmoles/g dry weight for sixteen values in different preparations of tissue. In experiments at 1 m*M*, the uptake was determined at 4 min only. In one preparation of tissue five replicates of the uptake of L-alanine gave the range 9.7–11.5 μmoles/g dry weight, while in another preparation of tissue four replicates of the uptake of L-isoleucine gave the range 11.0–15.5 μmoles/g dry weight.

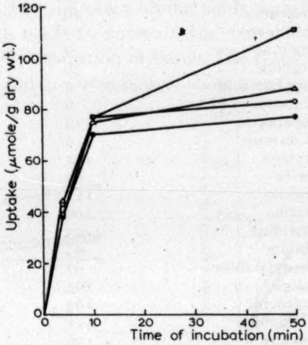

Fig. 1. Replicates of the uptake of L-alanine. Segments of small intestine incubated with L-alanine at 10 m*M*.

Since the variation between preparations is greater than the variation within a single one, it was thought best to make single determinations for each amino acid on several preparations of tissue with determinations for uptake of as many amino acids as possible on each. Up to thirteen amino acids have been compared on the one preparation of tissue.

Recovery of the amino acid taken up

To distinguish whether the observed uptake might arise by metabolic transformation of the amino acid rather than by its accumulation within the tissue, studies were made of the extent to which the amino acids were recoverable from the tissue after the uptake period. To allow the maximum effect of metabolism they were made after 50 min incubation.

Inspection of the results in Table III shows that there is considerable metabolism of L-ornithine, L-arginine, L-aspartic acid, L-glutamic acid and L-glutamine, while the remaining amino acids tested are fully recoverable within the limits of experi-

TABLE III

THE RECOVERY OF L-AMINO ACID DISAPPEARING FROM THE MEDIUM

A known wet weight (1.0–1.2 g) of tissue incubated for 50 min with L-amino acid at 10 mM, or without amino acid for control tissue. Recovery of amino acid disappearing from the medium determined as described in text.

Amino acid	*% recovery of amino acid disappearing from the medium*
Lysine	76
Lysine	95*
Ornithine	40
Ornithine	56*
Arginine	9
Arginine	36*
Aspartic acid	5
Glutamic acid	9
Asparagine	119
Glutamine	19
Glycine	102
Serine	84
Proline	112
Proline	109
Histidine	93
Alanine	93
Phenylalanine	97
Valine	104
Isoleucine	103
Methionine	111
Leucine	106

* Tissue washed with 1 % w/v Na_2CO_3 in 70 % ethanol.

TABLE IV

THE PERCENTAGE OF L-AMINO ACID DISAPPEARING FROM THE MEDIUM RECOVERED AS α-AMINO-NITROGEN IN VARIOUS AMINO ACIDS

Experimental details as described in text.

Amino acid incubated with tissue	*Recovery as % of the disappearance of incubated amino acid from the medium*					
	Arginine	*Ornithine*	*Aspartic acid*	*Glutamic acid*	*Glutamine*	*Alanine*
Arginine	9, 36	56, 26				
Ornithine	Nil	40				29
Aspartic acid			5	Nil		58
Glutamic acid			Nil	9		57
Glutamine				7	19	47

mental error. From Table IV it will be seen that the alanine appears as the main product containing α-amino nitrogen. From the limitations of the chromatographic separation used, it is not excluded that glycine is metabolised to serine, but conversion of L-serine to glycine is excluded.

In so far as comparisons are possible, the results agree with those of WISEMAN *et al.*[12, 13]. The complete recovery of L-asparagine and the substantial disappearance of L-glutamine are in contrast to findings for guinea pig intestine *in vitro*[14]. The absence of asparaginase activity in extracts of rat intestinal tissue has been noted[15].

The finding that lysine is strongly adsorbed on to ethanol-extracted tissue and that washing the tissue with sodium carbonate in aqueous ethanol increases the recovery of the positively charged amino acids suggests that an adsorption to anionic groups of the tissue may contribute to the value determined for the uptake of these amino acids.

Time-progress of uptake

In preliminary experiments time-progress curves were determined for the uptake of L-histidine at a number of concentrations. In all these curves the rate of uptake decreased in a similar manner with time, so that there was little increase in the total uptake after 20–30 min. For the other amino acids determinations at a single concentration (10 m*M*) were made to compare the time-progress of uptake with that of L-histidine in the same preparation of tissue. The results of two such experiments are presented in Figs. 2 and 3. These figures show two types of curve; those shaped similarly to the curves for L-histidine uptake and those showing a more continuous uptake with time. The amino acids, L-glutamine, L-arginine, L-glutamic acid and L-aspartic acid, showing the second type of curve are among those which are metabolised by the tissue. A summary of the results of these and a number of other similar experiments is presented in Table V as the averages of the ratios of uptake at 10 min to uptake at 4 min and of the ratios of the uptake at 50 min to uptake at 4 min. These other experiments confirmed that the amino acids which are not completely recoverable show a continued uptake, whereas those which are largely recoverable show a tendency to reach a point where net uptake ceases. An exception in the latter class, however, is L-lysine which shows a more continuous uptake than L-histidine. For those other amino acids which are not metabolised, the similarity in the shape of the curves is indicated by the similarity of the two sets of ratios.

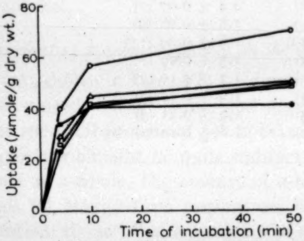

Fig. 2. The time-progress of uptake of various L-amino acids. Segments of small intestine incubated with L-amino acid at 10 m*M*. ○—○, L-histidine; ■—■, L-phenylalanine; △—△, L-methionine; □—□, L-leucine; ●—●, L-isoleucine.

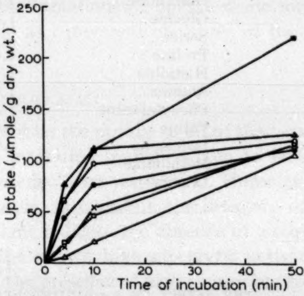

Fig. 3. The time-progress of uptake of various L-amino acids. Segments of small intestine incubated with L-amino acid at 10 m*M*. ▲—▲, L-asparagine; ○—○, L-histidine; ■—■, L-glutamine; ●—●, L-proline; □—□, L-glutamic acid; ×—×, L-arginine; △—△, L-aspartic acid.

The existence of a steady state, *i.e.* influx = efflux, after net uptake has ceased is suggested by results[7] on the release of accumulated L-histidine. In terms of a hypothesis of a constant rate of influx and a rate of efflux increasing with the amount of amino acid taken up, the similarity of the shapes of the curves would indicate

TABLE V

AVERAGE RATIOS OF THE UPTAKE AT 10 min TO UPTAKE AT 4 min AND OF UPTAKE AT 50 min TO UPTAKE AT 4 min FOR L-AMINO ACIDS AT 10 m*M*

Segments of small intestine incubated with L-amino acid at 10 m*M*. Sampled for determination of uptake at 4, 10 and 50 min. Ratios calculated for individual experiments. Individual ratios averaged and presented as the mean ± S.E.M., for given number of experiments.

Amino acid	*Average ratio* *Uptake at 10 min* *Uptake at 4 min*	*Average ratio* *Uptake at 50 min* *Uptake at 4 min*
Lysine	2.6 ± 0.50 (2)	3.2 ± 0.80 (2)
Ornithine	2.1 ± 0.44 (3)	2.9 ± 0.38 (3)
Arginine	2.3 ± 1.00 (2)	6.5 ± 0.05 (2)
Aspartic acid	2.6 ± 0.67 (3)	9.4 ± 2.99 (3)
Glutamic acid	2.3 ± 0.05 (2)	4.7 ± 0.90 (2)
Asparagine	1.5 (1)	1.7 (1)
Glutamine	2.0 ± 0.20 (2)	3.4 ± 0.20 (2)
Glycine	1.6 ± 0.10 (4)	1.7 ± 0.17 (4)
Serine	1.5 ± 0.10 (2)	1.9 ± 0.16 (2)
Proline	1.6 ± 0.10 (2)	2.4 ± 0.07 (2)
Histidine	1.7 ± 0.07 (9)	1.8 ± 0.08 (9)
Alanine	1.6 ± 0.05 (13)	1.8 ± 0.09 (13)
Phenylalanine	1.4 ± 0.10 (2)	1.5 ± 0.07 (2)
Valine	1.6 ± 0.00 (2)	1.7 ± 0.10 (2)
Isoleucine	1.8 ± 0.06 (3)	1.9 ± 0.17 (3)
Methionine	1.5 ± 0.00 (3)	1.9 ± 0.21 (3)
Leucine	1.5 ± 0.10 (2)	1.8 ± 0.10 (2)

TABLE VI

THE EFFECT OF 2,4-DINITROPHENOL ON THE UPTAKE OF L-AMINO ACIDS

Segments of small intestine incubated with L-amino acid (10 m*M*). Test experiments carried out with $2 \cdot 10^{-4}$ *M* 2,4-dinitrophenol, controls no 2,4-dinitrophenol. Test tissue preincubated for 10 min with $2 \cdot 10^{-4}$ *M* 2,4-dinitrophenol, control tissue preincubated for 10 min without 2,4-dinitrophenol. Sampled for determination of uptake at 4, 10 and 50 min. Uptake in presence of 2,4-dinitrophenol expressed as percentage of uptake in control.

Amino acid	*Uptake in the presence of $2 \cdot 10^{-4}$ M DNP (% uptake in control)*		
	4 min incubation	*10 min incubation*	*50 min incubation*
Lysine	86, 110	97, 84	94, 108
Ornithine	19, 80	51, 82	53, 77
Arginine	132	140	53
Aspartic acid	54	95	52
Glutamic acid	62	64	57
Asparagine	24	28	10
Glutamine	48	52	72
Glycine	42	38	26
Serine	25	52	48
Proline	60	42	37
Histidine	59, 74, 83	50, 69, 62	10, 71, 36
Alanine	58, 40	53, 40	26, 29
Phenylalanine	21	35	31
Valine	41	37	50
Isoleucine	33, 60	50, 64	46, 46
Methionine	27	49	50
Leucine	46	41	60

similar relationships between rate of efflux and amount of amino acid taken up for each amino acid. Such might be expected to be the case if the major part of efflux were in a water stream discharging the amino acid in solution at a rate proportional to the amount present internally. The similarity would seem unlikely to be the result of efflux by diffusion, or by reversal of the uptake process, since the rate of these processes might be expected to be more greatly affected by the differences in the properties of the different amino acids.

While the tendency to attain a steady state as a result of increased efflux seems the most probable interpretation of the curves observed, it is also possible that the rate of influx decreases. This might occur through damage to the tissue on prolonged incubation. An inhibition of uptake due to amino acid previously taken up seems unlikely[16]. The similar shape of the curves, indicated by similarity of the various ratios between the uptakes at different times, implies that the uptake is at the same stage for the different amino acids, so that determinations of uptake made for the same time of incubation should be comparable as equivalent measures of the rate of uptake.

The effect of 2,4-dinitrophenol on the uptake of amino acids

As shown in Table VI, 2,4-dinitrophenol inhibits the uptake of all of the L-amino acids tested, except lysine and arginine. The inhibition may be taken to indicate that the total process of net uptake requires energy from respiration. However, the requirement might be quite indirect; for example, to maintain the integrity of the tissue as a whole. The absence of inhibition might indicate the absence of a requirement for energy from respiration, but with the known high glycolytic activity of intestinal tissue[17, 18] a dependence of uptake on the presence of high energy phosphate compounds cannot be eliminated using 2,4-dinitrophenol alone. With these reservations, it might be inferred that, for L-lysine and perhaps L-arginine, the process of uptake does not have a requirement for useful energy from respiration. This requirement is indicated for the other amino acids tested, but for those which are not fully recoverable from the tissue no conclusion can be drawn as to whether the effect is on accumulation within the tissue or on metabolism. These results extend and confirm previous findings[4, 7, 14].

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THE UPTAKE OF AMINO ACIDS BY ISOLATED SEGMENTS OF RAT INTESTINE

II. A SURVEY OF AFFINITY FOR UPTAKE FROM RATES OF UPTAKE AND COMPETITION FOR UPTAKE

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SUMMARY

Average rates have been determined for the uptake by segments of rat small intestine of 17 amino acids at the concentrations of 1 mM and 10 mM. At 10 mM the order is such that the more lipophilic amino acids show the smaller rates, but at 1 mM the order is distinctly reversed, with the more lipophilic amino acids having the greater rates. On the assumption that MICHAELIS-MENTEN kinetics apply to the rate of uptake, MICHAELIS constants have been calculated from the average rates at 1 mM and 10 mM. From these constants, assuming that the amino acids compete for uptake by a MICHAELIS-MENTEN mechanism, predictions have been made of the effect of one amino acid on the other and have been compared with observed values. In general, the predictions agree well with the observed values, suggesting that the assumptions may be correct; but exceptions to the agreement suggest that L-lysine, and probably L-ornithine and L-arginine, may not compete for a common mechanism with the other L-amino acids. If the agreement is taken to implicate a rate-limiting, reversible combination with a common site in the uptake of most L-amino acids, then such a site shows a higher affinity for the more lipophilic amino acids. Limited studies with D-amino acids suggest that they may also combine with the site for uptake of L-amino acids, but have a lower affinity for it.

INTRODUCTION

The specificity of the active process for transport of amino acids by isolated intestine has not been clearly defined by previous work. WISEMAN[1,2] has given evidence of different rates for different amino acids in the transfer by isolated sacs of hamster small intestine. Since the study was carried out at a single concentration, information of the changes of the rate with concentration, from which an order of affinities might be inferred, is not available. With the techniques of both transfer[1] and uptake[3], and probably of absorption[4], the more lipophilic amino acids have the greater effectiveness as inhibitors of the transport, suggestive of a greater affinity. Agreement between the affinities intrinsic to rates of transport and inhibition of transport would suggest that the amino acids are combined only with common sites in the process of transport. The present paper represents an attempt to compare affinities for uptake and inhibition of uptake. The comparison has been made on the basis of MICHAELIS-MENTEN kinetics.

EXPERIMENTAL METHODS

The techniques used and the allowances for water exchange were the same as in the previous paper[5].

RESULTS

Comparisons of rates at different concentrations

From preliminary studies of the uptake at 4 min of several amino acids over a range of concentrations (Figs. 1, 2 and 3), it was decided to make a survey of rates of uptake at concentrations of 1 m*M* and 10 m*M*, using the uptake at 4 min as a measure of the rate of uptake. Individual determinations were made for each amino acid on several preparations of tissue, comparing as many amino acids as possible on the one tissue. The average values for the uptakes at 1 m*M* and at 10 m*M* are presented in Table I. It is apparent that at both concentrations L-amino acids possessing similar charges and oil-water partition characteristics show similar rates of

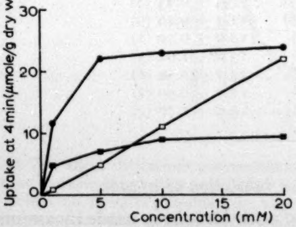

Fig. 1. The effect of concentration on the rate of uptake of L-lysine, L-glutamic acid and L-isoleucine. Segments of small intestine incubated with amino acid at the given concentrations. ■—■, L-lysine; □—□, L-glutamic acid; ●—●, L-isoleucine.

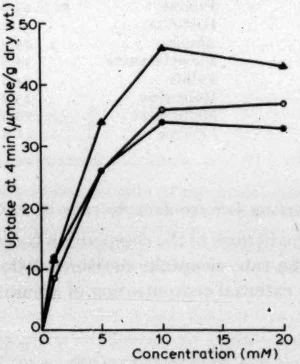

Fig. 2. The effect of concentration on the rate of uptake of L-histidine, L-alanine and L-isoleucine. Segments of small intestine incubated with amino acid at the given concentrations. ○—○, L-histidine; ▲—▲, L-alanine; ●—●, L-isoleucine.

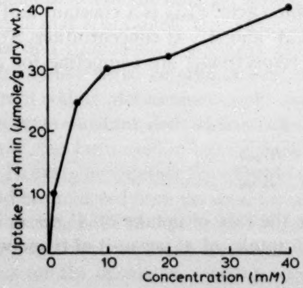

Fig. 3. The effect of concentration on the rate of uptake of L-phenylalanine. Segments of small intestine incubated with [^{14}C]amino acid at the given concentrations. Change in concentration of amino acid in the external solution measured by direct plating and counting in a gas flow counter.

uptake, except in so far as metabolism of the amino acids may have affected the determination[5]. For the amino acids without a third charged group at pH 7.4 the general trend at 10 mM is that the more lipophilic amino acids have the lower rates of uptake; the order being closely similar to that for transfer at 20 mM (see ref. 1, 2). The trend at 1 mM, however, is reversed.

TABLE I

RATES OF UPTAKE OF L-AMINO ACIDS

Segments of small intestine incubated with amino acid at given concentration, uptake at 4 min taken as a measure of the rate of uptake. Results presented as the mean ± S.E.M. for the bracketed number of experiments.

Amino acid	*Uptake at 10 mM (µmole/g dry wt.)*	*Uptake at 1 mM (µmole/g dry wt.)*
Lysine	10.0 ± 1.96 (5)	6.8 ± 1.37 (3)
Ornithine	23.2 ± 2.72 (4)	5.3 ± 2.40 (2)
Arginine	16.7 ± 1.80 (4)	7.8 ± 1.41 (3)
Aspartic acid	14.4 ± 2.05 (3)	1.6 ± 1.02 (3)
Glutamic acid	13.6 ± 2.17 (5)	1.8 ± 0.53 (3)
Asparagine	57.3 ± 8.49 (3)	8.6 ± 0.97 (3)
Glutamine	51.0 ± 6.32 (4)	11.3 ± 2.20 (2)
Glycine	35.7 ± 4.08 (9)	2.7 ± 0.77 (3)
Serine	54.7 ± 9.13 (4)	8.1 ± 1.11 (3)
Proline	43.6 ± 1.87 (5)	7.8 ± 0.65 (2)
Histidine	41.1 ± 2.58 (15)	7.35 ± 0.85 (8)
Alanine	45.3 ± 1.57 (16)	11.3 ± 0.49 (9)
Phenylalanine	35.5 ± 1.50 (2)	10.9 ± 0.70 (2)
Valine	34.7 ± 3.88 (4)	13.6 ± 1.92 (3)
Isoleucine	25.2 ± 2.06 (9)	12.7 ± 0.54 (8)
Methionine	24.0 ± 1.88 (5)	13.7 ± 0.50 (2)
Leucine	21.5 ± 3.50 (2)	13.9 ± 1.70 (2)

Comparison between data for rates of uptake and for inhibition of uptake

Predictions of the competition between amino acids have been made by assuming that the rate of uptake measured follows MICHAELIS-MENTEN kinetics with respect to the external concentration of amino acid. That is, the rate of uptake,

$$v = \frac{V_{\max}S}{K_m + S} \qquad (1)$$

where S is the external concentration of the amino acid, $V_{\max}$ is a constant and K_m is the MICHAELIS constant. If two amino acids, A' and A'', at concentrations S' and S'' with MICHAELIS constants K'_m and K''_m, respectively, are competing for such a mechanism, the relationship,

$$\frac{v'_{A''}}{v'} = \frac{K'_m + S'}{K'_m + S' + \dfrac{K'_m S''}{K''_m}} \qquad (2)$$

derived from THORNE[6], will apply. Here $v'_{A''}$ is the rate of uptake of A' per unit of tissue in the presence of A'' and v' is the rate of uptake of A' per unit of tissue when alone.

The results presented in Figs. 1–3 suggest that it may be reasonable to assume MICHAELIS-MENTEN kinetics in considering the inhibition of uptake. As a partial test of the assumptions of MICHAELIS-MENTEN kinetics and competition for a common site in the uptake of L-histidine and L-isoleucine, the uptakes of L-histidine in the presence of varying concentrations of L-isoleucine were determined, and compared (Fig. 4) with values predicted from the average control uptake and the MICHAELIS constants derived from the rates of uptake at 1 m*M* and 10 m*M* (see Table II). The agreement gives support for the assumptions.

Table II presents values for the MICHAELIS constants calculated from the average rates in Table I, together with the predicted uptake of L-histidine at 10 m*M* in the presence of other amino acids at 10 m*M*. For comparison the experimental values of AGAR *et al.*[3] for uptake of L-histidine in the presence of other amino acids are included. Calculation of a MICHAELIS constant from the rates of uptake for glycine yields a negative value, since the rate at 1 m*M* is less than 0.1 the rate at 10 m*M*. If a constant is calculated from the data for its inhibitory effect on the uptake of L-histidine, the value 34 m*M* is obtained. This value coupled with the average rate at 10 m*M* predicts a rate of 4.5 μmoles/g dry weight at 1 m*M* as compared with the average value of 2.7 found for three determinations. It is possible that the errors involved in the different values determined could account for this discrepancy. The value of the MICHAELIS constant derived from the effect of glycine on L-histidine uptake, that is, 34 m*M*, has been used in later calculations for glycine.

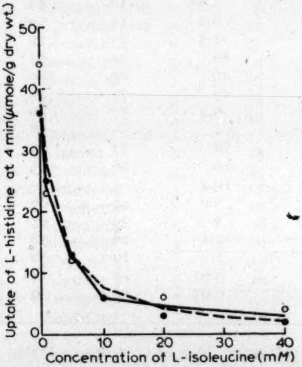

Fig. 4. The effect of various concentrations of L-isoleucine on the rate of uptake of L-histidine. Segments of small intestine incubated with L-histidine at 10 m*M* and the given concentrations of L-isoleucine. Results presented for experiments on two different preparations of tissue. ●—●, O—O, experimental points; ———, average of experimental points; -----, values predicted from the MICHAELIS constants and the average uptake of L-histidine in the absence of L-isoleucine.

To examine whether the effect of amino acids on the uptake of other amino acids would be predicted from the data for rate of uptake, some further series of experiments were performed. The results for combinations of the amino acids, L-lysine, L-glutamic acid, L-histidine, L-alanine and L-isoleucine, are given in Table III. Table IV gives values for the uptake of L-amino acids in the presence of 10 m*M* L-isoleucine. The

TABLE II

MICHAELIS CONSTANTS FOR THE UPTAKE OF L-AMINO ACIDS WITH PREDICTED AND OBSERVED UPTAKES OF L-HISTIDINE IN THE PRESENCE OF THE AMINO ACIDS

MICHAELIS constants calculated from the rates of uptake at 1 m*M* and 10 m*M* (Table I). Values for the uptake of L-histidine at 10 m*M* in the presence of L-amino acid at 10 m*M* expressed as a percentage of the uptake in control (L-histidine alone at 10 m*M*). Predicted values calculated from the MICHAELIS constants as described in text, experimental values from AGAR *et al.*[3]. No corrections for water exchange.

Amino acid	*Michaelis constant (mM)*	*Uptake of L-histidine in presence of other amino acid (% control uptake)*		
		Predicted	*Experimental*	
			at 15 min	*at 45 min*
Lysine	0.55	10	83	76
Ornithine	6.0	54	—	—
Arginine	1.5	23	79	77
Aspartic acid	80	94	81	87
Glutamic acid	26	84	110	105
Asparagine	17	77	74	73
Glutamine	6.4	57	76	81
Glycine	—	—	78	87
Serine	18	77	84	78
Proline	10	66	75	74
Histidine	10.4	—	—	—
Alanine	5.0	49	66	57
Phenylalanine	3.3	39	45	49
Valine	2.1	28	38	43
Isoleucine	1.2	19	30	22
Methionine	0.91	15	14	23
Leucine	0.65	11	9	21

results of further studies of inhibitions, including some on the uptake of L-isoleucine, are given in Table V. In each of these Tables, the inhibited uptake is expressed as a percentage of the control uptake. The average inhibited uptake is also given in μmole/g dry weight to allow an estimate of the absolute magnitude of the percentage values. Thus in some cases apparently large differences between the predicted and experimental percentage values would require only small differences in absolute uptake.

Predictions involving L-lysine, L-ornithine and L-arginine show some marked disagreement with experimental values (Tables II, III and IV). Adsorption and, for L-ornithine and L-arginine, metabolism[5] of the amino acid may play a part in the observed uptake of these three amino acids, but, from the small inhibition of uptake by L-isoleucine, uptake by a process involving combination with a site common with L-isoleucine is unimportant.

L-aspartic and L-glutamic acids have low rates of uptake (Table I) and low affinities in uptake (Fig. 1, Tables II and III). The non-recovery of these two amino acids from the tissue[5] means that the uptake may substantially represent metabolism rather than accumulation within the tissue, so that the comparisons of data may not be valid. However, qualitatively, the strong inhibition by L-isoleucine and L-histidine (Table IV) suggests that an uptake, involving the same site as does the uptake of L-isoleucine, is a rate limiting step in the removal from the medium.

TABLE III

THE UPTAKE OF SOME L-AMINO ACIDS IN THE PRESENCE OF ONE ANOTHER

Segments of small intestine incubated 4 min with the given concentrations of the amino acids under study. Controls contained the amino acids singly. Predicted values calculated from the MICHAELIS constants (Table II) as described in text.

Amino acid studied, concentration (mM)		*Additional amino acid, concentration (mM)*		*Uptake in the presence of additional amino acid*		
				Experimental		*Predicted*
				(µmole/g dry wt.)	*(% control uptake)*	*(% control uptake)*
Glutamic acid	20	Isoleucine	2	5.0	26	52
Glutamic acid	5	Isoleucine	5	1.5	31	22
Glutamic acid	5	Lysine	5	5.1	104	12
Glutamic acid	20	Lysine	20	21	110	5
Glutamic acid	5	Glutamic acid	5	5.5*	112*	86*
Lysine	5	Glutamic acid	5	11	153	99
Lysine	20	Glutamic acid	20	21	162	98
Lysine	20	Isoleucine	5	9.5	73	91
Lysine	5	Isoleucine	5	6.5	93	73
Lysine	5	Lysine	5	4.5*	64*	57*
Isoleucine	5	Lysine	5	20	87	36
Isoleucine	5	Glutamic acid	5	22	96	97
Isoleucine	5	Alanine	5	24	92	84
Isoleucine	5	Histidine	5	25	96	91
Isoleucine	5	Isoleucine	5	11.5*	52*	55*
Isoleucine	5	Isoleucine	5	17*	65*	55*
Alanine	5	Isoleucine	5	6.4	19	32
Alanine	5	Histidine	5	29	85	80
Alanine	5	Alanine	5	23*	67*	67*
Histidine	5	Alanine	5	20	77	60
Histidine	5	Isoleucine	5	2.6	10	26
Histidine	5	Histidine	5	18*	69*	75*

* These values based on half the uptake at 10 m*M*, using the uptake at 5 m*M* as control.

TABLE IV

THE UPTAKE OF L-AMINO ACIDS IN THE PRESENCE OF L-ISOLEUCINE

Segments of small intestine incubated 4 min with amino acid (10 m*M*) and L-isoleucine (10 m*M*). L-isoleucine omitted in the control. Predicted values calculated from the MICHAELIS constants (Table II) as described in text.

Amino acid	*Uptake in the presence of 10 mM L-isoleucine*		
	Average uptake (µmole/g dry wt.)	*Individual experiments (% control uptake)*	*Predicted values (% control uptake)*
Lysine	8.5	69, 75	69
Ornithine	15	65, 74	24
Arginine	19	108, 132	48
Aspartic acid	6.5	44, 33	12
Glutamic acid	—2.5	—18, —25	12
Asparagine	12	20, 30	16
Glutamine	9	29, 16	24
Glycine	7	14, 23, 31, 26	14
Serine	5	13, 15	16
Proline	5	16, 9	19
Histidine	6	17, 13	19
Alanine	13	43, 31	26
Valine	7	20, 29	41
Isoleucine	14*	50*, 52*	53*
Methionine	16	60, 77	59

* These values are based on half the uptake at 20 m*M*, using the uptake at 10 m*M* as the control.

TABLE V

THE UPTAKE OF SOME L-AMINO ACIDS IN THE PRESENCE OF ANOTHER AMINO ACID

Segments of small intestine incubated 4 min with the amino acid studied (10 m*M*) and the given concentration of an additional amino acid. No additional amino acid in control. Predicted values calculated from the MICHAELIS constants (Table II) as described in text.

Amino acid studied	*Additional amino acid present,*	*Concentration (mM)*	*Average uptake (μmole/g dry wt.)*	*Individual experiments (% control uptake)*	*Predicted values (% control uptake)*
Isoleucine	L-proline	10	24	83	90
Isoleucine	L-alanine	10	17	95	82
Isoleucine	L-valine	10	8	43, 33	66
Isoleucine	L-methionine	10	12	50, 45	42
Phenylalanine	L-phenylalanine	10	16*	53*	57*
Aspartic acid	L-histidine	10	6	27	53
Glycine	L-histidine	10	6	21	58
Valine	L-histidine	10	32	75	85
Histidine	DL-α-amino-octanoic acid	2	15	44, 34	—

* These values based on half the uptake at 20 m*M*, using the uptake at 10 m*M* as control.

The two amides L-asparagine and L-glutamine show higher affinities and much greater rates of uptake than the corresponding acids (Tables I and II). For L-asparagine there is good agreement between the predicted and observed effects (Tables II and III). For L-glutamine, agreement with prediction might not be expected, since this amide is metabolised by the tissue[5]; but since the uptake of L-glutamine is strongly inhibited by L-isoleucine it seems likely that combination with a site common to other amino acids is a rate limiting step in the uptake process.

For the remaining L-amino acids studied the experimental results obtained show a reasonable agreement with the predictions. The large number of experiments with L-histidine and L-isoleucine, separately and together, and with other amino acids almost all give data in good agreement with the predictions. The general trend of agreement between the predicted and experimental values, except for the amino acids, lysine, arginine and ornithine, provides further support for the two assumptions of MICHAELIS-MENTEN kinetics and a common site in the uptake of most L-amino acids.

Considered as inverse measures of the affinity, the MICHAELIS constants for the process of uptake (Table II) show the affinity of this site to be greater for the more lipophilic L-amino acids. An interesting indication that this high affinity for lipophilic L-amino acids is not restricted to the naturally occurring amino acids comes from the effectiveness of DL-α-amino-octanoic acid as an inhibitor of the uptake of L-histidine (Table V).

The importance of the external concentration of amino acid (amino acid in the medium) rather than the internal concentration (amino acid taken up) in inhibiting the uptake of a second amino acid is shown by the experiment presented in Fig. 5. The results show that L-isoleucine taken up during a prior incubation is not an effective inhibitor of the uptake of L-histidine, whereas L-isoleucine present in the medium is an effective inhibitor. The results would appear to exclude the importance of retention sites within the tissue in the mechanism of uptake of L-histidine.

The uptake of and inhibition by D*-amino acids*

Some investigations of the uptake of certain D-amino acids are presented in Table VI. Experiments were carried out on the effect of various amino acids at 10 m*M*, including some D-amino acids, on the uptake of L-histidine at 0.5 m*M*. The experiments were unsatisfactory in that the correction for release of histidine from the tissue makes up a large proportion (about 60 %) of the uptake at this concentration, so that relatively small variations in this release may cause considerable errors in the uptake determined. The results are presented in Table VII as being of qualitative value in making comparisons of the effectiveness of these amino acids as inhibitors of the uptake of L-histidine.

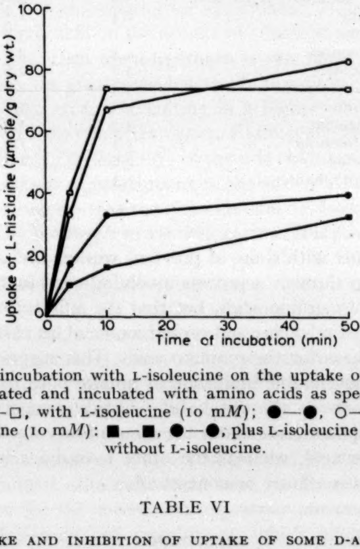

Fig. 5. The effect of preincubation with L-isoleucine on the uptake of L-histidine. Segments of small intestine preincubated and incubated with amino acids as specified below. 10 min preincubation: ■—■, □—□, with L-isoleucine (10 m*M*); ●—●, ○—○, without L-isoleucine. Incubation with L-histidine (10 m*M*): ■—■, ●—●, plus L-isoleucine (10 m*M*); □—□, ○—○, without L-isoleucine.

TABLE VI

THE UPTAKE AND INHIBITION OF UPTAKE OF SOME D-AMINO ACIDS

Segments of small intestine incubated 4 min with D-amino acid at the given concentrations. The effects of 10 m*M* L-isoleucine and 2,4-dinitrophenol[5] determined as for L-amino acids.

Amino acid	*Uptake at 10 mM (μmole/g dry wt.)*	*Uptake at 1 mM (μmole/g dry wt.)*	*Uptake at 10 mM in presence of DNP (% control uptake)*	*Uptake at 10 mM in presence of 10 mM L-isoleucine (% control uptake)*
D-serine	8, 13	—	—	50, 38
D-threonine	3, 7	—	—	0, −29
D-histidine	6, 7	—	—	−16, −14
D-alanine	8, 13, 16	5.5	100	62, 44
D-phenylalanine	6, 2	0.6	—	—
D-valine	10, 11	—	—	—
D-isoleucine	7, 12	2.0, 2.7	171, 42	—
D-methionine	25, 24, 21	2.0, 3.3	19	16, 29
D-leucine	23, 22	—	—	—

TABLE VII

THE EFFECTS OF D-AMINO ACIDS AND SOME OTHER AMINO ACIDS ON THE UPTAKE OF L-HISTIDINE

Segments of small intestine incubated 4 min with 0.5 m*M* L-histidine plus an additional amino acid at 10 m*M*. Additional amino acid omitted in the control. Values corrected for the blank released from tissue incubated in medium without amino acid and estimated as 2.5 and 2.4 μmoles of histidine/g dry wt. in Expt. 1, and 2.2 and 2.1 μmoles of histidine/g dry wt. in Expt. 2.

Additional amiuo acid	*Uptake of L-histidine (μmole/g dry wt.)*	
	Expt. 1	*Expt. 2*
Control (L-histidine alone)	3.9, 4.4	3.2, 3.7
α-amino-isobutyric acid	2.0	2.2
L-2,4-diamino-butyric acid	3.9	3.0
Glycine	2.4	1.5
D-serine	4.1	3.4
D-threonine	4.3	3.5
D-alanine	3.7	2.6
D-phenylalanine	3.4	2.1
D-valine	4.3	1.9
D-isoleucine	3.7	3.2
D-methionine	1.5	1.0
D-leucine	2.6	1.8
D-tryptophane	1.3	1.5

The results, together with those of previous workers[3,7,8], indicate that D-amino acids may be taken up through a process involving combination with the site concerned in the uptake of L-amino acids, but that the affinity for the site is relatively low. The rapid uptake of D-leucine and D-methionine at 10 m*M* suggests a particular importance of the process for these amino acids. This suggestion for D-methionine is supported by the considerable inhibition of uptake both by L-isoleucine and 2,4-dinitrophenol. The effects on the uptake of L-histidine suggest that D-methionine, D-leucine and D-tryptophane have about the same affinity for the site as have glycine and L-amino-isobutyric acid, whereas the other D-amino acids and L-2,4-diamino-butyric acid have a lower affinity or none at all.

DISCUSSION

The conclusions drawn for the uptake of the individual amino acids may be summarised to state that the process involves combination with a site which appears to be available to all of the L-amino acids tested, with the possible exceptions of lysine, ornithine and arginine. There is evidence that some D-amino acids also combine with the site. The higher affinity for the more lipophilic L-amino acids is in agreement with previous results on the uptake, transfer and absorption of L-histidine[3,4], and on competition in the transfer of amino acids[1].

An important aspect of the results obtained is the reversal of the order of rates with change in concentration. The effect that the rate of uptake at high concentrations is in the inverse order to the affinity, and at low concentrations is in the same order as the affinity has been predicted by WILBRANDT[9] for uptake by carrier transport. In such a system the rate would be determined by the degree of saturation of the carrier and the net rate of dissociation from it. The first of these would be increased, and the second decreased, with greater affinity for the carrier, so lowering the maximum rate on complete saturation of the carrier system, but raising the rate at concentrations well below saturation. The similar time-progresses of uptake of amino acids[5], or the concentration of amino acids against a gradient by intestinal tissue[1-3], are not predicted by the mechanism proposed by WILBRANDT[9], but are explicable by relatively simple modifications of it.

On the data derived in the present work, suggestions may be made as to the nature of the site involved in the uptake of amino acids by small intestine. It is proposed that the site has specific points of attachment for the L-amino and L-carboxyl groups, with an adjacent lipophilic region situated so as to allow concurrent attachment of the lipophilic side chains of the L-amino acids. Since glycine attaches to the site, a side chain is not an essential for attachment. Moreover, if the inhibitory effect of L-amino-isobutyric acid on the uptake of L-histidine can be taken to indicate combination with the site, then the α-hydrogen is also non-essential for the attachment. For L-amino acids, the greater the length of the carbon chain the more strongly is it held to the lipophilic area, so resulting in a higher affinity for the site and a slower dissociation from it to effect transport. Hydrophilic groups on the side chain will have the reverse effect. The flexibility of the side chain may also be of importance in determining the readiness of attachment to the site.

It is reasonable to suggest that the site functions to determine the rate of transport of the amino acid by binding it to a mobile carrier. It is possible that the transport of amino acids may be the unique function of the site, or that the site may also be the binding centre of an enzyme involving amino acids, *e.g.* a peptidase or transaminase. It is conceivable that the location of an enzyme on a mobile carrier could "adsorb" an amino acid and, without the presence of an acceptor molecule (*e.g.* keto acid in the case of transaminase), move it from point A and release it at point B, without structural change. Previous information[1-5] and that in the present paper is consistent with the existence of a mobile carrier for the movement of amino acids into the tissue and of some further process for the movement from the tissue (possibly as a solute in a water stream) at a rate proportional to the amount in the tissue. The concentration gradient obtained would be a resultant of these two processes. A plausible suggestion for the mechanism of carrier movement is that it involves the flow and vesiculation of cell membranes, which might support the specific carrier sites[10, 11].

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THE SIMILAR PROPERTIES OF TRYPTOPHAN SYNTHETASE AND A MUTATIONALLY ALTERED ENZYME IN *NEUROSPORA CRASSA*[*]

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SUMMARY

A method has been developed for the stabilization and further purification of tryptophan synthetase from *N. crassa*. The sedimentation constant, diffusion coefficient and approximate molecular weight of the enzyme from strain C-84 have been determined. A marked similarity in physical properties was found between the enzyme and an antigenically related protein in one tryptophan-requiring mutant. These properties include behavior during purification, elution pattern during DEAE cellulose column chromatography, and sedimentation velocity constants. The results support the hypothesis that the antigenically related proteins in the td mutants are in fact genetically altered tryptophan synthetase molecules.

INTRODUCTION

Certain mutant strains of *Neurospora crassa* (td mutants), which require tryptophan for growth and which lack the enzymic ability to synthesize L-tryptophan from L-serine and indole, contain proteins (CRM) which are antigenically very similar to wild-type Tsase[1-3]. Since these mutations represent distinct genetic alterations[4-6], a comparison of the properties and ultimately the structure of the normal enzyme and the CRM proteins offers one approach to understanding the types of changes in a protein molecule which can occur as a consequence of mutations within a single genetic locus.

Studies with Tsase from *N. crassa* have been severely handicapped in the past by the lability of the enzyme, limiting the degree of purification which could be attained. This is a report of the stabilization of Tsase and the subsequent purification of the enzyme and a CRM from one td mutant. The proteins have been compared in several of their physical properties and the results strongly support the contention that the CRM proteins represent genetically altered tryptophan synthetase molecules.

Abbreviations: Tsase, tryptophan synthetase; TCA, trichloroacetic acid; PALP, pyridoxal phosphate; EDTA, ethylenediaminetetraacetate; DEAE, diethylaminoethylcellulose; GSH, reduced glutathione.

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MATERIALS AND METHODS

Preparation of crude extracts

Tryptophan synthetase was obtained from *N. crassa*, strain C-84 (see ref. 7), a histidine mutant, grown on minimal medium[8] supplemented with L-histidine (53 mg/l). The enzyme from C-84 resembles Tsase obtained from several wild-type strains (74A, 5297, and 5256A) in those properties which have been examined, viz. stability and antigenic activity.

CRM was obtained from *N. crassa*, strain td_2^9, a tryptophan-requiring mutant grown on minimal medium supplemented with DL-tryptophan (150 mg/l). Both strains were grown in 10 l carboys with vigorous aeration for 50 to 80 h, harvested by filtration through cheesecloth, washed with distilled water, promptly frozen, and lyophilized. Dried mycelia were powdered with a mortar and pestle and the crude extracts were prepared by stirring the powder into cold potassium phosphate buffer (0.1 *M*, pH 7.8). After standing for 20 min at 2–5° the slurry was centrifuged at 15,000 × *g* for 20 min and the supernatant solution, containing the enzyme, was decanted.

Tryptophan synthetase assay

Tryptophan synthetase activity was measured by following indole disappearance colorimetrically, using the *p*-dimethylaminobenzaldehyde reaction[10]. Tryptophan formation by purified enzyme preparations was verified by chromatography in a butanol–acetic acid–water system using a Precision Chromatofuge. One enzyme unit (TSU) is defined as that amount of enzyme which will catalyze the conversion of 0.1 µmole of indole to tryptophan during a 60-min incubation period at 37°. The reaction mixtures contained 0.1 *M* K_2HPO_4, pH 7.8, indole (0.5 µmole), DL-serine (80 µmoles), and pyridoxal phosphate (0.96 µmoles), with ethylenediaminetetraacetate (EDTA, 2 µmoles) added to protect the enzyme.

CRM assay

CRM activity was measured using rabbit anti-CRM serum[3]. A highly specific anti-CRM was prepared by injecting rabbits with a fractionated preparation of td_2-CRM which had been further purified by precipitation of non-CRM proteins with rabbit anti-td_1 serum[3] (strain td_1 contains neither Tsase nor CRM and is similar to mutant td_2 at loci other than the td locus[10]). Agar diffusion data indicate that the protein remaining after precipitation of td_2-CRM preparations with anti-td_1 is capable of inducing the formation of antisera which contain anti-CRM as the major component[11]. Samples containing CRM in 0.1 *M* potassium phosphate buffer, pH 7.8 were pre-incubated with antiserum for 15 min at 0°. The quantity of uncombined anti-CRM remaining after pre-incubation was determined by the Tsase-inhibition reaction[3]. One unit of CRM is defined as the amount of CRM which will combine with that quantity of anti-CRM necessary to inhibit one tryptophan synthetase unit. It has been previously shown that the reaction of anti-CRM with CRM and Tsase is complete and irreversible under the conditions of the assay[3]. All CRM values are based on quantitative absorption curves such as the one shown in Fig. 1.

Protein assay

Specific activities of Tsase and CRM were calculated as tryptophan synthetase

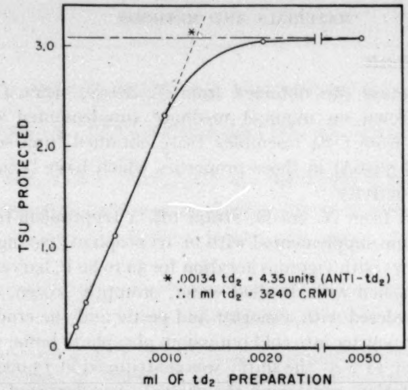

Fig. 1. CRM assay. CRM diluted with 0.1 M K-PO$_4$, pH 7.8, containing BSA 1.0 mg/ml was added to tubes containing 4.35 units of anti-td$_2$ in 0.15 ml. The mixture was incubated for 5 min at 37° and recooled to 3°. 5.50 units of crude C-84 Tsase in 0.10 ml was added with sufficient buffer to bring tubes to 0.90 ml. After 15 min, the substrate–coenzyme mixture was added in 0.75 ml, and residual enzymic activity assayed. Control enzyme–antibody tubes contained 1.85 TSU, indicating that they were in the non linear portion of the enzyme–antibody curve. This accounts for the difference between maximum TSU Protected (3.08) and Anti-td$_2$ added (4.35). CRM activity per/ml was calculated by extrapolating the linear portion of the curve to 3.08 TSU, the value at which all anti-CRM was absorbed.

units (TSU) and CRM units (CRMU)/mg protein. Protein was measured colorimetrically by the method of Lowry *et al.*[12], spectrophotometrically by the ratio of absorption at 260 mμ and 280 mμ[13], or turbidometrically by the absorption at 600 mμ after the addition of TCA to a final concentration of 3% (see ref. 14).

Stability assays

The stability of Tsase at 2–5° was studied by storing aliquots of a given preparation in stoppered test tubes. At intervals duplicate tubes were removed and the reaction mixture for the Tsase assay was added directly to these tubes. In other experiments 1-ml aliquots of Tsase were dialyzed in the cold. Individual sacs were removed at intervals and duplicate 0.15-ml samples were assayed for residual enzymic activity.

Heat stability was measured by immersing test tubes containing aliquots of Tsase or CRM in a water bath at the desired temperature. The Tsase reaction mixture was added directly to the tubes after they were cooled to 0°.

Purification of Tsase and CRM

A method of purification was developed for Tsase and applied without modification to td$_2$-CRM. All operations were performed in a cold room, refrigerated centrifuge or ice bath at temperatures below 3°. Low temperature is essential for the preservation of maximal activity during the first two steps of purification. All solutions used in purification as well as in assays were made up in glass-distilled water. Low conductivity water was used since it is known that Tsase is inhibited by certain trace metals[10].

During a typical purification 768 ml of crude extract was obtained from 50 g of powdered mycelia extracted in 800 ml of phosphate buffer. *Step I*: The pH was adjusted from 7.4 to 6.9 with 17 ml of 1 *N* acetic acid, and 93 ml (14 ml /100 ml extract) of a 1.5 % protamine sulfate solution was added rapidly drop by drop with stirring over a 15-min period. After standing for 15 min the samples were centrifuged at 15,000 × *g* for 20 min.

Step II: A supernatant solution of 790 ml was obtained and the pH was adjusted from 6.9 to 5.7 with 1 *N* acetic acid. Saturated ammonium sulfate solution (3°) was added dropwise over a 40-min period to give 30 % saturation. Small amounts of a 10 % (v/v) ammonium hydroxide solution were added to maintain the pH at 5.60 ± 0.05 during the ammonium sulfate addition. The final mixture was stirred gently for 25 min and centrifuged at 15,000 × *g* for 20 min. The supernatant solution was discarded and the precipitate taken up to a volume of 50 ml in a mixture containing 0.1 *M* potassium phosphate buffer (K-PO_4) pH 7.8, $1 \cdot 10^{-4}$ *M* PALP and $1 \cdot 10^{-2}$ *M* EDTA.

Step III: To this slightly turbid solution were added 8 ml of 0.2 *M* DL-serine, 15 ml of $4 \cdot 10^{-3}$ *M* PALP, 15 ml of 0.1 *M* EDTA, 40 ml of 0.5 *M* K-PO_4 buffer pH 7.0, and 22 ml of 0.1 *M* K-PO_4, pH 7.8. The resulting 150 ml were brought to 54° over a 15-min period and then maintained at 54° for 5 min. After cooling in an ice bath, the heavy precipitate was centrifuged for 20 min at 15,000 × *g*. The resulting 142 ml of clear supernatant solution was first dialyzed for 5 h at 3° against 1500 ml of glass-distilled water containing $1 \cdot 10^{-5}$ *M* PALP and $1 \cdot 10^{-3}$ *M* EDTA, followed by a 10-h dialysis against 1500 ml of 0.01 *M* K-PO_4, pH 7.8 containing the same concentrations of PALP and EDTA.

Step IV: To the dialyzed material were added 5 ml of $4 \cdot 10^{-3}$ *M* PALP and 3 ml of 0.1 *M* EDTA. The pH was adjusted to 5.8 with 1 *N* acetic acid. Saturated ammonium sulfate was added dropwise to give 25 % saturation. (pH was maintained at 5.8 with NH_4OH as in *step II*). The slightly turbid mixture was stirred gently for 15 min and centrifuged at 15,000 × *g* for 20 min. The precipitate was discarded. The pH was adjusted to 5.0 with 1 *N* acetic acid and saturated ammonium sulfate was added to give 31 % saturation. After gentle stirring for 5 min, the mixture was centrifuged for 20 min at 15,000 × *g* and the precipitate taken up in 0.1 *M* K-PO_4, pH 7.8 containing $1 \cdot 10^{-4}$ *M* PALP and $1 \cdot 10^{-2}$ *M* EDTA. The sample was dialyzed for successive 6- and 12-h periods against 0.01 *M* K-PO_4, pH 7.8 containing $1 \cdot 10^{-5}$ *M* PALP and $1 \cdot 10^{-3}$ *M* EDTA.

Step V: Details of the preparative fractionation on a DEAE cellulose column are similar to those given below for analytical chromatography. However, for preparative work, a 1.8 · 10 cm column was used and elution was effected rapidly within a 4 to 5 h period under a pressure of approximately 6 pounds/in^2. A non-linear gradient was obtained by adding 100 ml of mixture "2" to a constant volume mixing chamber initially containing 80 ml of mixture "1" (see below).

Analytical chromatography

Commercial DEAE cellulose was repeatedly washed with 0.3 *M* KOH and distilled water, followed by 0.3 *M* KH_2PO_4 and distilled water. After adjusting the pH to 7.8, with 0.3 *M* KOH it was washed with several liters of 0.01 *M* K-PO_4 pH 7.8 containing $1 \cdot 10^{-3}$ *M* EDTA and suspended in this buffer 2 % (w/v). Columns

(0.8 × 10 cm) were filled with the suspension, allowed to settle under gravity, packed briefly under a pressure of 3 pounds/in^2 and decanted. The final column was washed with 20 to 40 ml of mixture "1" (0.01 M K_2HPO_4, pH 7.8 with $1 \cdot 10^{-5}$ M PALP and $1 \cdot 10^{-3}$ M EDTA). The samples of Tsase or CRM which had been dialyzed against mixture "1" were put on the column under a pressure of 2 pounds/in^2. A linear gradient was developed by adding 60 ml of mixture "2" (0.5 M K_2HPO_4, pH 7.8, with $1 \cdot 10^{-4}$ M PALP and $1 \cdot 10^{-2}$ M EDTA) to a mixing chamber containing an equal volume of mixture "1" in such a way that the volumes of the two chambers remained approximately equal throughout. Elution took place over 12 to 15 h at a rate of 8–12 ml/h.

Sedimentation velocity

Sedimentation velocities of Tsase and CRM were determined either by measuring the decrease in enzymic or antigenic activity in the separation cell of TISELIUS[15] or by conventional optical technics, employing a Spinco Model E Ultracentrifuge. Calculations of sedimentation velocity in the separation cell were made assuming stable sedimentation with a well defined boundary[16]. This assumption was confirmed by the experimental results. All sedimentations were made in 0.1 M K-PO_4 pH 7.8 with $1 \cdot 10^{-4}$ M PALP and $1 \cdot 10^{-3}$ M EDTA.

Diffusion coefficient

The diffusion coefficient for Tsase was measured by the diaphragm-cell method of NORTHROP AND ANSON[18,19]. Two cells (volumes 4.42 and 5.19 ml) were run simultaneously. They were calibrated immediately prior to this experiment with 1 N HCl assuming a diffusion coefficient of 1.54 cm^2/day at 4° (see ref. 20). Partially purified Tsase (1016 TSU/ml and 5.7 mg protein/ml) in 0.1 M K_2HPO_4 buffer, pH 7.8 containing $1 \cdot 10^{-4}$ M PALP, $1 \cdot 10^{-3}$ M EDTA and $1 \cdot 10^{-4}$ M serine was dialyzed twice against the same buffer-stabilizer solution. An identical buffer-stabilizer solution was used for the external bath (20 ml) into which Tsase diffused. Bovine serum albumin (BSA-100 µg/ml) was added to the bath to protect the purified enzyme against inactivation during dilution. The effect of BSA can be seen in Table I. Tsase assays were performed in quadruplicate or sextuplicate.

In order to confirm the stability of Tsase during the diffusion process (83 h)

TABLE I

LOSS OF ACTIVITY ON DILUTION IN ABSENCE OF BOVINE SERUM ALBUMIN

The table gives the apparent activity (TSU/ml) of a partially purified preparation of C-84 Tsase (specific activity 280 TSU/mg protein) when various buffer mixtures were used to dilute the sample in preparation for assay. The three diluents shown are: (a) 0.1 M K-PO_4, pH 7.8, alone, (b) K-PO_4 with $1 \cdot 10^{-4}$ M PALP and $1 \cdot 10^{-2}$ M EDTA, and (c) K-PO_4 with BSA 1.0 mg/ml. The assays themselves were carried out in the presence of PALP, EDTA and BSA. Other experiments showed that washing of glassware with 5 N nitric acid did not prevent loss of activity. Gelatin but not protamine sulfate or heparin will prevent loss. After initial loss during dilution, the activity remained stable in K-PO_4, EDTA, and PALP for 170 h at 3°.

	KPO₄	*TSU in* *KPO₄, PALP, EDTA*	*KPO₄ BSA*
Dilution 1:10	680	680	800
Dilution 1:100	470	470	800

and to average out errors due to fluctuations in the temperature of the Tsase assay (approx. 6%/ degree at 37°), each diffusate sample was assayed 3 times at daily intervals. Although slight variations were observed in each set of three assays, there was no evidence for enzymic inactivation. Therefore, each set of three values was combined and the average value used. All of the initial enzymic activity was quantitatively recovered from the baths and the cells at the end of the experiment.

RESULTS

Stabilization of Tsase

YANOFSKY[10] reported that partial stabilization of Tsase at 5° could be achieved by the addition of GSH and PALP. More than 90% of the activity of crude enzyme preparations is preserved at 3° for 160 h by adding PALP, DL-serine and EDTA. Tsase from four different strains of *N. crassa* (5256A, 5297a, 74A and C-84) were found to behave similarly in this respect. Virtually complete stabilization of the partially purified Tsase from C-84 is found under the same conditions for 215 h. PALP, EDTA and serine each increase the stability of Tsase in crude extracts when used separately, but the rate of inactivation of crude enzyme and the degree of protection afforded by each compound alone is somewhat different for the four strains (Table II).

TABLE II

A COMPARISON OF THE STABILITIES OF TSASE FROM SEVERAL STRAINS OF *N. crassa*

Crude enzyme was diluted with 0.1 *M* K-PO_4, pH 7.8 to approx. 10 TSU/ml and 0.40-ml aliquots placed in tubes with additives and K-PO_4 to make 0.55 ml. The tubes were stored covered with Saran wrap at 3°. Duplicate tubes were assayed at 0, 4, 18, 72 and 140 h. The values listed in the table are the percent of 0 h control activity remaining at 100 h as read from the plotted curves.

Buffer mixture	*Strain*			
	5256A	*5297a*	*74A*	*C-84*
(1) 0.1 *M* K-PO_4, pH 7.8	32%	40%	—	13%
(2) (1) + 0.01 *M* EDTA	50	42	37	58
(3) (1) + 4 × 10^{-4} *M* PALP	70	87	76	74
(4) (1) + 0.02 *M* DL-serine	49	55	27	47
(5) (3) + (4)	98	91	88	92
(6) (2) + (3)	91	87	85	95
(7) (2) + (3) + (4)	99	92	92	95

At temperatures above 50° neither PALP nor EDTA significantly alter the rate of inactivation of partially purified Tsase. L-serine or D-serine is required in addition to PALP to protect against inactivation at 55–56° for 30 to 40 min. Partially purified td_2-CRM is also stabilized at 55° by PALP and serine. Alanine and glycine have some stabilizing effect on the enzyme, while phenylalanine, cysteine, threonine, aspartic acid, and tyrosine are of little or no value. Potassium propionate and acetate decrease heat stability as does pyruvate even in the presence of pyridoxamine phosphate.

Pyridoxal phosphate is specifically required to stabilize the enzyme both at low temperatures and above 50°. Pyridoxine, pyridoxal, pyridoxamine and pyridoxamine phosphate failed to protect. They have also been shown to be ineffective as coenzymes for Tsase activity[10].

Although phosphate buffer has been used exclusively in the work reported here, the stability of Tsase in glycylglycine, veronal, potassium maleate and Tris-HCl (0.1 *M*, pH 7.8) was examined. Enzymic activity is lost in Tris-HCl (50% in 17 h during dialysis) and Tris itself inhibits the enzymic assay. Tris may compete with the enzyme for PALP, since Tris-PALP mixtures develop an intense yellow color with a spectrum similar to PALP-serine in the 350 to 450 mμ range. The activity in glycylglycine is also somewhat unstable (25% loss in 160 h). In veronal or maleate the enzyme was stable for 160 h but the assay itself is slightly inhibited by these buffers.

Purification of Tsase and CRM

Results of the purification of Tsase and CRM are shown in Table III and Fig. 2. It is evident that the method developed for Tsase is effective for purifying CRM. Step III is possible because PALP and DL-serine stabilize partially purified Tsase at 55° while considerable denaturation of other proteins occurs. Heat treatment of crude extracts has not proved to be a satisfactory purification step. The conditions for the two ammonium sulfate fractionations are based on data derived from the precipitation of Tsase at different pHs. (Fig. 3). Although maximum total activity is precipitated in Step II at pH 5.9, the specific activity of the enzyme recovered by pH 5.6 precipitation is higher.

TABLE III

PURIFICATION OF C-84 TRYPTOPHAN SYNTHETASE AND CRM FROM MUTANT td_2

	C-84 Tsase			*td_2-CRM*		
	Total activity	*Specific activity**	*% recovery*	*Total activity*	*Specific activity**	*% recovery*
Crude enzyme	38,000 TSU	3.2	100	40,300 CRMU	4.5	(100)%
Step I						
Protamine sulphate	36,000	8.1	95	44,000	7.5	100%
Step II						
Ammonium sulphate pH 5.6	23,000	53	61	33,000	53	75
Step III						
Heat	20,700	190	55	32,200	230	73
Step IV						
Ammonium sulphate pH 5.0	17,000	430	45	23,000	360	52
DEAE column**	9,750	880	26	13,000	630	30

* Specific activity: units of Tsase or CRM/mg of protein as measured by absorption at 260 mμ and 280 mμ.

** These figures apply to a pool of the three fractions from each column which contained the highest specific activity. Considerably higher % recovery was obtained from the total of all column fractions: viz., 13,000 TSU for the Tsase column and 17,000 CRMU for the CRM column. Furthermore, the peak specific activity for each column is higher than this average.

Some purification can also be achieved by absorption on alumina gel at pH 5.8 and elution at higher pH and ionic strength. Purification by absorption and elution from Ca-PO_4 gel has been reported[10].

No gradients other than ionic strength have been employed for the DEAE column purification step.

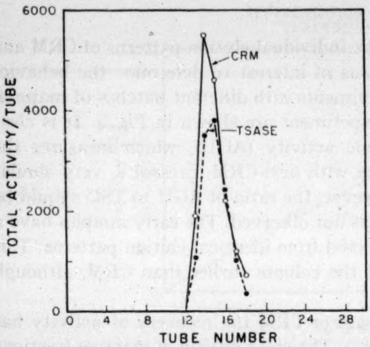

Fig. 2. Elution patterns for preparative DEAE cellulose columns. The figure shows the remarkable similarity between the elution patterns for Tsase and CRM during the purification of each on separate preparative DEAE columns. Only tubes 12 to 18 were assayed in detail. All tubes were given a preliminary screening which would have detected 100 units per tube and no evidence was obtained for a second peak of activity. See text for details of method.

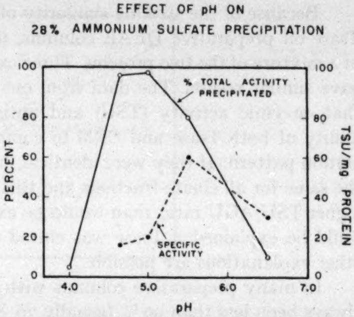

Fig. 3. Precipitation of C-84 Tsase with ammonium sulfate. Crude C-84 which had been treated with protamine sulfate (step II) was divided into 25-ml aliquots containing 1000 TSU. The pH was adjusted with 1 *N* acetic acid at 3°. Saturated ammonium sulfate was added dropwise to a final saturation of 28% while maintaining the pH within 0.05 units variation. The precipitate was centrifuged at 15,000 × *g* for 20 min and resuspended in 0.1 *M* K-PO$_4$, pH 7.8 with $1 \cdot 10^{-4}$ *M* PALP and $1 \cdot 10^{-2}$ *M* EDTA. Protein assays were done by the method of LOWRY[12] at dilutions where the PALP did not give a significant color.

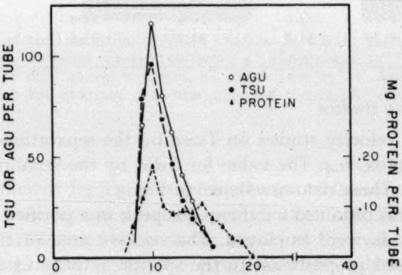

Fig. 4. Elution patterns for a mixture of Tsase and CRM from analytical DEAE cellulose column. A mixture containing 810 enzymic units (TSU) and 700 CRMU (total = 1180 Antigenic units* (AGU)) and 4.80 mg protein in 8.0 ml was chromatographed by methods detailed in the text. In tubes 8 through 20, 425 TSU, 427 AGU, and 1.06 mg protein were recovered, with less than 1.0 TSU or AGU or 0.02 mg protein in any of the other tubes. Tube volumes were 3.95 ± 0.05 ml. Tubes 39 and 40 were eluates from washing the column with mixture "2" undiluted, in an effort to remove any residual protein.

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* The enzyme–antigen activity ratio greater than 1.00 which was found in tube 9 (E/A 1.21) may reflect the high ratio (E/A 1.69) of the purified Tsase preparation used in the original mixture. Such high ratios have been seen frequently and may simply indicate a lower enzyme–antigen ratio in the Tsase preparation used to standardize the anti-CRM serum.

Analytical chromatography

Because of the striking similarity of the individual elution patterns of CRM and Tsase on preparative DEAE columns, it was of interest to determine the behavior of a mixture of the two proteins. Three experiments with different batches of material gave similar results. The data from one experiment are shown in Fig. 4. It is clear that enzymic activity (TSU) and antigenic activity (AGU), which measures the ability of both Tsase and CRM to combine with anti-CRM, present a very similar elution pattern. If they were identical, however, the ratio of AGU to TSU should be the same for all eluate fractions and this was not observed. The early samples have a higher TSU/AGU ratio than would be expected from identical elution patterns. This could be explained if Tsase was eluted off the column earlier than CRM, although other explanations are possible.

In many preparative columns with Tsase or CRM the recovery of activity has always been less than 90 % (usually 70–80 %). The cross-mixing of inactive fractions from the column has failed to demonstrate any separation of materials which can be reconstituted to yield active enzyme. In addition, if two components were being separated by the column procedure, one might expect to find an increase in activity when mixing early and late eluate samples. No increase has been found (Table IV).

TABLE IV

RESULTS OF MIXTURES OF COLUMN ELUATE FRACTIONS

Assays contained 0.10 ml from each of the two tubes in a given mixture. Assays performed in duplicate. For details, see text.

	TSU		*AGU*	
	Observed	*Calculated*	*Observed*	*Calculated*
Tubes 8 + 11	21.0	20.9	21.4	22.3
Tubes 9 + 12	31.8	31.7	33.5	32.2

Sedimentation velocity studies

Sedimentation velocity studies on Tsase by the separation-cell technic gave an S_{20} for Tsase of approx. 6.4. The value for CRM by the separation-cell method was higher, approx. 6.9. These data are shown in Fig. 5.

An S_{20} of 6.0 was obtained for the major peak in a purified preparation of Tsase when optical methods were employed. The relative area of this peak in different preparations appeared proportional to the specific activity of the enzyme. The less well-defined, smaller maximum has an S_{20} of approximately 1.8. The S_{20} for CRM based on optical measurements was 6.7. In order to compare the sedimentation velocities of CRM and Tsase under identical conditions, the sedimentation of a mixture of approximately equal amounts of purified CRM and Tsase was examined optically. The sedimentation pattern of the mixture was quite similar to that of Tsase alone and the major peak had an S_{20} of about 5.9. There is a slight widening of this peak, which is compatible with a difference of about 10 % (5–15 %) in the sedimentation velocities of CRM and TSase. This is in agreement with the difference observed when Tsase and CRM were compared separately. The sedimentation patterns for Tsase, CRM and the Tsase-CRM mixture are shown in Fig. 6.

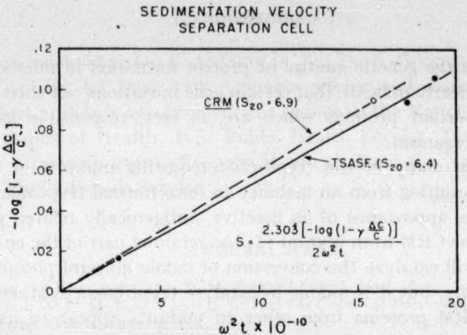

Fig. 5. This figure plots data corrected to 20°. The viscosity of the solvent has been assumed to be that of pure water. For details of method see text[16].

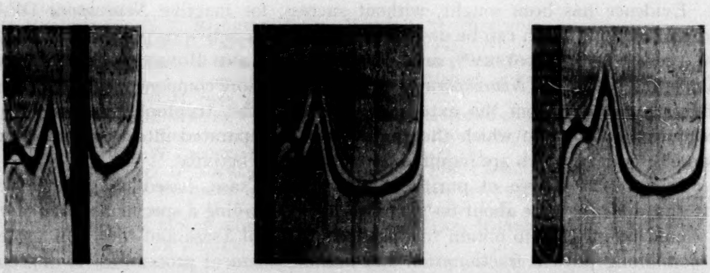

Fig. 6. Optical patterns of sedimentation velocity studies. A. CRM, 2600 CRMU and 3.6 mg protein/ml. Separation cell. 118 min, 42,040 rev./min., 4°, diaphragm 25°. B. Tsase, 4600 TSU and 6.6 mg protein/ml; 116 min, 42,040 rev./min, 6°, diaphragm 25°. C. Mixed Tsase and CRM-equal volumes of A and B mixed; 116 min, 42,040 rev./min., 8°, diaphragm 25°.

Diffusion coefficient

The diffusion coefficient for Tsase at 4° in the two cells was 0.0230 and 0.0211 cm²/day as calculated from six successive 8-h periods after a constant rate of diffusion had been reached. The difference between the results of the two cells is statistically significant, representing some systematic error. Average values for the diffusion coefficient corrected to 20° are $D = 0.0365$ cm²/day or $4.22 \cdot 10^{-7}$ cm²sec.

Molecular weight of Tsase

Calculated from $S_{20} = 6.4 \cdot 10^{-13}$ sec and D_{20} $4.2 \cdot 10^{-7}$ cm²/sec obtained on the same preparation, the molecular weight of Tsase is approx. 140,000. The errors from the data themselves place a 95 % confidence limit of ± 12 % on this figure. However, since the choice of a partial molar volume (0.74) for the enzyme was of necessity arbitrary, and since the values for the sedimentation constant obtained by optical and separation-cell methods differ slightly, this molecular weight estimate of Tsase must be considered to have confidence limits of 15–20 %.

DISCUSSION

Current studies on the genetic control of protein formation in microorganisms and higher organisms clearly indicate that certain gene mutations can elicit the formation of structurally aberrant proteins which are, in fact, responsible for the mutant phenotype of the organism.

In the case of many of the tryptophan-requiring mutants of *N. crassa*, the metabolic block, resulting from an inability to form normal tryptophan synthetase, is coupled with the appearance of an inactive, antigenically related protein, CRM. It is known that the CRM from mutant td_2 has retained part of the enzymic activity of Tsase, viz., it will catalyze the conversion of indole glycerol phosphate to indole and triose phosphate, but it is unable to catalyze tryptophan synthesis from indole and serine[21–25]. CRM proteins from other td mutants appear to have their own characteristic enzymic properties, yet they all lack normal catalytic activity[24, 26]. Thus it appears that localized, specific damage to a protein can occur as a consequence of particular site mutations within a gene locus.

Evidence has been sought, without success, for inactive *Neurospora* DEAE-protein fractions which can be used to reconstitute an active tryptophan synthetase. RACHMELER AND YANOFSKY[25], and WUST, DEMOSS AND BONNER[32] have also been unable to separate the *Neurospora* Tsase into two or more components. These findings differ significantly from the extensive studies on the tryptophan synthetase of *Escherichia coli*[27–31], in which the enzyme can be separated into two components, A and B, both of which are required for full enzymic activity.

The present degree of purity of *Neurospora* Tsase, based on sedimentation patterns, appears to be about 60 % in preparations having a specific activity of 700. It should be possible to obtain further purification of Tsase and the CRM proteins by combining protein fractionation and immunochemical procedures. A molecular weight of about 140,000 is large for convenient structural analysis, but it may be possible to fragment the protein to a more suitable size by enzymic digestion. In conclusion, the results of these experiments indicate a marked similarity in physical properties between Tsase from strain C-84 and CRM from mutant strain td_2. These include (a) a similar behavior in the purification procedure, (b) similar if not identical DEAE-elution patterns for enzymic and antigenic activities in a mixture of CRM and Tsase, and (c) sedimentation velocity constants which are the same within experimental error (approx. 10 %). These findings, in addition to the antigenic similarity between CRM and Tsase[3], strongly support the view that the td_2-CRM protein represents genetically altered tryptophan synthetase. The close similarity between these two proteins does not imply that the antigenically related proteins in other td mutants of *N. crassa* resemble Tsase to this degree. In fact, in at least one case, the mutation has been of sufficient magnitude to prevent recognition of the protein by any of the presently available means[1,3]. In other td mutants, as mentioned earlier, specific, characteristically modified CRM proteins are formed[24,26]. Consequently, it seems most likely that a variety of mutational changes can occur within the td locus, and that the ultimate configuration and properties of the enzyme tryptophan synthetase reflect these changes.

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CONFIGURATIONAL TRANSITION IN LYSOZYME

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SUMMARY

Experimental techniques have been developed for conveniently measuring the temperature dependence of rotation of proteins using relatively small quantities of material. Results of rotation-temperature studies on lysozyme in aqueous urea solutions are presented and compared to earlier results with ribonuclease. Both the ribonuclease and the lysozyme undergo an inverted configurational transition in concentrated urea solutions; *i.e.*, some protein goes from a "denatured" to a "native" state when the temperature is increased. The reversal of the transition was also studied and found to be first order in lysozyme and to have a large activation energy.

INTRODUCTION

During recent years a great deal of work has been done in studying the physicochemical properties of synthetic polypeptides[1]. The studies were carried out not only because these newly available compounds are intrinsically interesting but also because it was hoped they might be satisfactory model compounds for the naturally occurring proteins. In many ways the latter hope has been borne out.

We have recently published a study of ribonuclease[2] which was undertaken to see if, in a sense, the proteins could be used as model compounds for synthetic polypeptides. In particular the work was initiated to see if it was possible to observe an effect DOTY AND YANG found[3] for PBGA in a mixed solvent and termed by them an "inverted transition". They found that on raising the temperature of the PBGA it went from a less ordered to a more ordered state. In a protein such an inverted transition would correspond to renaturing a partially denatured protein by elevating its temperature. As is well-known this is certainly not the normal behavior of proteins.

Denaturation is normally defined in operational terms and for convenience we chose to define it in terms of the same easily measurable physical characteristic used by DOTY AND YANG viz., the optical activity[4-6]. Using this criterion for denaturation we demonstrated the existence of inverted transitions for ribonuclease in concentrated urea solutions. Admittedly, ribonuclease with its twenty odd amino acids and four disulfide cross links is a very complex "model compound" for PBGA though it does have the advantage of having a very well defined molecular weight. On the other hand it is a good "model protein".

Abbreviation: PBGA, polybenzylglutamate.

The optical rotation is a measure of the average environment of the optically active centers of the protein. Thus any changes in structure of the polypeptide "backbone" of the protein (where most of the asymmetric centers are located) will result in a change in the optical activity. For this reason the more descriptive term "configurational transition" will be used to characterize the type of denaturation to be discussed in this paper. Such a transition may be induced by raising the protein temperature, adding a strong hydrogen bond former to the protein solution or by a combination of these methods.

A number of questions were raised by the study on ribonuclease, but before returning to them we decided to initiate the present experiments on lysozyme. There were several reasons for doing this, but the most important was the desire to develop procedures for working with smaller quantities of material since we were using as much as 300 mg for a single experiment with ribonuclease. In addition we wished to see what similarities there are between the behavior of ribonuclease and lysozyme.

In this paper only those effects on lysozyme which result from the presence of a strong hydrogen bond former (urea) will be considered. Consequently, the experiments were all carried out at essentially the same pH (near 6) and at moderately high ionic strength (0.06 to 0.1).

EXPERIMENTAL

Armour crystalline egg white lysozyme Lot D-638040 was used for all of the experiments. This lot contained 9.0 % moisture as determined by drying at 100°. The solutions were all made up in 0.1 *M* potassium chloride. This solution was either added directly to the protein or else to the required amount of solid urea. None of the solutions were buffered. The urea was Baker and Adamson reagent grade recrystallized once from ethanol.

Rotations were measured with a precision Rudolph polarimeter with a rocking polarizer. Unless indicated otherwise all of the measurements were made at the strong mercury 365 mμ line. In the earlier experiments with ribonuclease all measurements of optical activity were made at 546 mμ rather than the traditional sodium D line since proteins have a higher specific rotation at this wavelength. In order to reduce the sample size the logical step was to go to still shorter wavelengths. In principle it should be possible with proteins to obtain increasingly great specific rotations to about 200 mμ, but in practice it is not feasible to work below 300 mμ because of the strong absorbance of the protein tyrosine, phenylalanine and tryptophan groups. In addition since light scattering increases markedly with decreasing wavelength, scattering losses can be serious. At present the 365-mμ line seems to be a satisfactory compromise.

The first measurements of the temperature dependence of rotation were made in a commercial (Rudolph) 1 dm glass polarimeter tube with a 3 mm bore. Light fluctuations discussed earlier are not as serious in the 3 mm tube as in the 8.5 mm tube used previously. Normally 1.5 ml of 1 % protein solution was prepared, so 15 mg of material was used for each experiment. It was found necessary to observe a number of precautions in studying the temperature dependence of rotation. Quartz windows should not be used because they exhibit a large temperature dependence of rotation with some hysteresis. Even with glass windows large changes (*e.g.*, 0.2°) were occasionally observed on heating polarimeter tubes to 80°. These rotations were probably the result of stresses set up in the glass because the differential expansion of the glass and metal in the tubes was not being taken up by the rubber gaskets. Several experiments were carried out in which the tubes were cycled through temperature changes between room temperature and 80°, and it was found that when large changes did occur it was only on the first cycle. For this reason these polarimeter tubes were routinely heated and cooled before making measurements at varying temperatures.

Because of these difficulties a new type of polarimeter tube was devised which proved much more satisfactory for this work. It was made from a rod of polymethylmethacrylate 2.99 cm in diameter and 10 cm long. A 3 mm wide slot was milled the length of the rod to a depth of 1.64 cm with a round bottomed tool. At first glass windows were cemented to the ends, but it proved more satisfactory to simply stick them on with silicone grease since birefringence effects were observed in the cemented windows when heated. Two tapered pins were used at each end of the tube to keep the windows from sliding down. The top surface of the tube was milled flat to permit the use of a flat piece of plastic for a cover. Solutions in tubes of this type respond very sluggishly to temperature changes in the constant temperature compartment of the polarimeter so the solution temperature was measured directly with a Veco 41A11 glass covered thermistor in a bridge circuit. Apart from the slow response to temperature changes these tubes have proven to be superior in almost all ways to the commercial jacketed capillary tubes. They are easy to fabricate, fill, clean and assemble. Normally 1.5 ml of solution is used in a 1 dm tube with a 3 mm slot, but this can be reduced to approx. 1.0 ml. Shortly before completing this work a polarimeter tube of this type was made from a solid rod of titanium. It has a more rapid temperature response and can be used at higher temperatures.

While using these tubes it was found that the simplest and most satisfactory way to clarify protein solutions was to filter them through a 0.5 μ Millipore filter with a syringe adaptor.

Viscosity measurements were made in Cannon-Ubbelohde semi-micro dilution viscometers (Size 25) having flow times of about 450 sec with water at 25°. The viscometers were calibrated from 25 to 60° with water. Since dust particles were troublesome the solutions were centrifuged, but the author would recommend the use of Millipore filters instead.

One of the most serious problems in making viscosity measurements was with frothing. By the judicious use of reduced pressure on the third tube of the Ubbelohde viscometer as well as on the capillary tube, it was possible to keep bubble formation to a minimum. In addition a thin gold wire was inserted in the viscometer in such a way that the end was just above the upper reference line. Many bubbles could be broken by raising and lowering them past this wire.

A Spinco Model E ultracentrifuge was used for several measurements discussed below. A Beckman Model G pH meter was used for all pH measurements.

RESULTS

Fig. 1 shows the rotation*-temperature curves for lysozyme in 0.1 *M* potassium chloride and in 8 and 10 *M* urea solutions. The latter solutions were prepared by adding

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[*] In all cases in this paper when the word "rotation" is used it means levorotation.

0.1 *M* potassium chloride to the required amount of solid urea and consequently have a somewhat lower ionic strength. The urea solution was then added to a sample of protein.

These curves were obtained by slowly raising the temperature and observing the rotations. Several hours were required to complete each experiment in the figure and under these conditions the rotations were only partially reversible (except in the case of 6 *M* urea to be discussed later). Two experiments in the absence of urea are shown in the drawing. In the first a freshly prepared lysozyme solution was heated from 25 to 67° and no appreciable change was observed in the rotation. The observed value at 25° is in excellent agreement with that recently reported by JIRGENSONS[7]. It was not possible to make measurements above 67° as the solutions became turbid. This turbidity is not caused by the precipitation of the lysozyme but by the precipitation of an impurity. That it is an impurity is shown by the fact that if a solution of lysozyme is heated and then filtered it does not become turbid on reheating to the same temperature. In addition the sedimentation pattern of the unheated solution shows a distinct (but very small) fast-moving peak. A one percent solution of lysozyme made up in 0.004 *M* mercuric chloride lacks the small peak in the sedimentation pattern and is slightly turbid when first prepared. Thus it appears that the impurity is precipitated by mercuric ions.

From an examination of the experiments in urea solutions (to be discussed below) it appeared that the thermal transition for lysozyme in 0.1 *M* potassium chloride should be half completed at 78°. After removing the impurity it was possible to make the required measurements in the titanium polarimeter tube. The results are shown in the second 0 *M* curve in Fig. 1. Measurements were not made beyond 82° as it was not possible to go to higher temperatures at the time the experiment was performed. However, the results clearly show that the transition is half-completed near 78° as predicted. With a modification of the experimental arrangement it became possible to go to higher temperatures, but it was found that the rotation only slowly increased on increasing the temperature. In order to carry out measurements through the entire transition it will be necessary to use a modified experimental set-up as evaporation becomes a serious problem when the solutions are heated for long periods at these temperatures.

To remove the impurity prior to these measurements the following procedure was used. 1 % lysozyme solutions in 0.1 *M* potassium chloride were heated 5 min at 90°. The small amount of cooked-egg-white-like material that formed was spun down in a clinical centrifuge. Since the exact lysozyme concentration was no longer known it was estimated by assuming a specific rotation of 201°; *i.e.*, it was assumed the protein remaining in solution was in its native state. It was possible to calculate the specific rotation of the protein remaining in solution by estimating the weight of the precipitate. The calculated specific rotation at room temperature was closer to 220 than to 201° suggesting some irreversible change in the heated protien. For this reason attempts are now being made to purify the lysozyme by a less drastic procedure.

Two additional experiments were performed in 4 and 6 *M* urea but are not shown in Fig. 1. In the 4 *M* solution it was not possible to complete the measurements on the transition since the impurity began to precipitate. However, it was possible to quite accurately estimate the temperature for the middle of the transition as 62.5°.

The 6 M experiment is especially interesting in that the rotation was completely reversible although the solution was heated from 13 to 75° during a 6-h interval. The transition was half complete at 56.2°. In several separate experiments rotation-temperature curves were obtained for lysozyme in 8 M urea over a period of several hours. At the completion of the experiments the solutions were allowed to stand overnight at room temperature. In all cases the final rotations were close to 230°. However, it is possible to obtain complete reversibility by heating these solutions for short periods of time. For example, on heating such a solution for 5 min at 80° and then suddenly cooling to 25° the rotation will return to its initial value in less than 15 min. (During this 5-min interval the rotations do increase to the values expected but it is difficult to obtain accurate measurements.) Unlike the experiments in 10 M urea the measurements in 8 M solutions were quite reproducible. Several experiments gave results that were identical within experimental error.

The reason for this irreversibility is not clear, though the possibility of oxidation by air during the heating appears to have been ruled out by the following experiments. Two solutions of lysozyme in 8 M urea were prepared that differed only in that one was saturated with nitrogen and the other with oxygen. Both were heated to 80° for 1 h and then cooled to room temperature. The levorotation of both solutions had increased about 60°.

Experiments performed in 10 M urea solutions give a variety of curves displaced vertically from one another; *i.e.*, the temperatures of the minima and maxima are essentially unchanged. For example Fig. 2 shows the results obtained in another experiment under conditions very similar to those of Fig. 1. Initially the levorotation was 238° at room temperature. When the temperature was raised the levorotation increased to a maximum of 284.5° and then started to decrease (total time elapsed between minimum and maximum temperature readings was 2 h). The solution was then quickly cooled to 25° and left overnight. The following day the upper curve was obtained when the sample was reheated. This solution was then cooled and diluted with 0.1 M potassium chloride to bring the urea concentration to 5 M. In less than 5 min the levorotation was 223°. Thus the lysozyme in the 5 M solution had apparently largely reverted to a native-like state as judged by the marked drop in levorotation and the large change in rotation on reheating.

Fig. 1 also includes the results of an experiment in which the viscosity of a lysozyme solution in 8 M urea was measured at various temperatures. The concentration of the protein was about 3 % and 1 ml of solution was used. The purpose of the experiment was to learn how close a parallelism existed between changes in viscosity and rotation. Since the rotation and viscosity curves have the same sigmoid shape the viscosity data were fit to the rotation data by equating the distance between the minima and maxima of the curves. As can be seen from the figure the correlation between viscosity and rotation changes in the transition region is striking.

It was found previously that when 7 M urea solutions of ribonuclease at 50° were suddenly cooled the levorotation increased rapidly before slowly returning to smaller values. A similar effect was looked for with lysozyme and first observed in the 10 M urea solution. If such a solution is rapidly cooled from 52° to 25° the rotation observed initially is on an extension of the negative slope of the temperature-rotation curve as indicated by a dotted line in Fig. 1. The rotation will not return to its original value on cooling if it remains at elevated temperatures for a long period. However, if

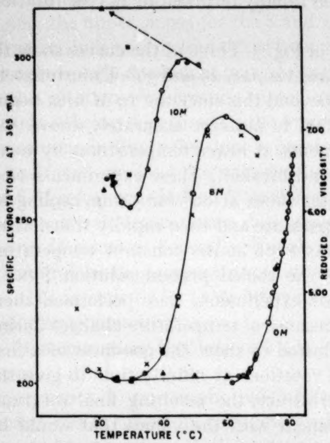


Fig. 1. Temperature dependence of levorotation and viscosity of lysozyme in 0, 8 and 10 *M* urea. (All solutions approximately 0.1 *M* in potassium chloride.) △ and ⊙ represent two separate experiments in 0 *M* urea. The crosses represent viscosity data in 8 *M* urea. The symbols ⊡, ▽ and △ near the 10 *M* curve represent reversal data at 30, 25 and 22° respectively. (See text and Fig. 3.)

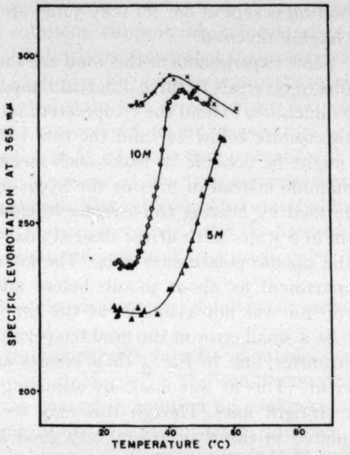
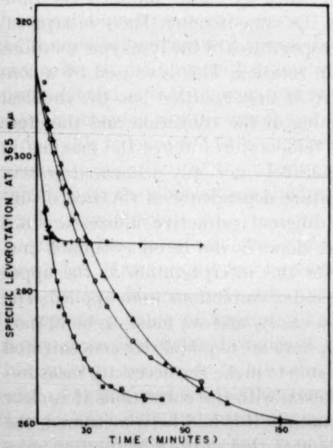


Fig. 2. Temperature dependence of levorotation of lysozyme in 10 and 5 *M* urea (plus approximately 0.1 *M* potassium chloride). ⊙, Freshly prepared solution of lysozyme in 10 *M* urea. ×, Same solution after standing overnight at room temperature. △, Same solution after diluting to 5 *M* in urea.



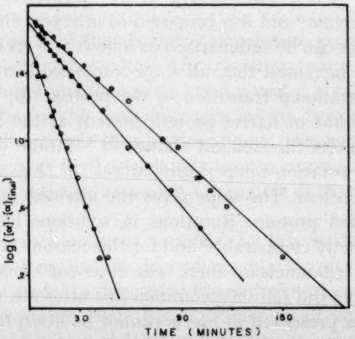


Fig. 4. First order plot of reversal data of Fig. 3.

Fig. 3. Time dependence of levorotation of lysozyme in 10 *M* urea after heating for 5 min and then returning to 22° △, ⊙ 25°; ⊡ 30°. + indicates a reversal at 25° but in the presence of mercuric chloride.

a solution is kept at 80° for only 5 min and then rapidly returned to 25° the rotations are largely reversed.

Some experiments of this kind are shown in Fig. 3. Three of the curves show the results of reversals to three different temperatures viz., 22, 25 and 30°. Unfortunately, it is difficult to extend the temperature much beyond this since the 10 M urea begins to precipitate below 22° and the rate is too fast to measure accurately above 30°. (It might be possible to make such measurements at lower temperatures by using formamide instead of urea as the hydrogen bond breaker.) These experiments were performed by heating the samples in closed test tubes at 80° for 5 min, cooling for 1 min in a water bath at the desired final temperature and then rapidly transferring to the plastic polarimeter tube. The latter was kept in the constant temperature compartment for about 30 min before adding the cooled protein solution. Since a thermistor was not available at the time these experiments were performed there may be a small error in the final temperature because of temperature changes during the transfer, etc. In Fig. 4 these results are plotted to show the goodness of a first order fit. (This fit was made by adjusting the rotations at infinite time to give the best straight lines. Though this may seem arbitrary, the resulting final rotations calculated in this way are in very good agreement with the values that would be estimated from Fig. 3). From the intercept the initial rotation can be calculated and these values are shown in Fig. 1. Two of the values are on the extension of the upper slope. Fig. 3 also includes an experiment in which mercuric chloride was present at a concentration of 0.004 M. The presence of the mercuric ions apparently partially blocks the reversal process.

DISCUSSION

General interpretations

The results of Fig. 1 are qualitatively the same as those obtained for ribonuclease[2] and it is proposed to interpret them in the same manner. These interpretations can be summarized as follows: (a) As the temperatures of the lysozyme solutions are increased they all show a marked increase in rotation. This is caused by a configurational transition in the protein. (b) The 10 M urea solution has the smallest amount of native protein present at the beginning of the transition and therefore exhibits the smallest change in rotation. (c) At temperatures above the maxima in the rotation-temperature curves the slopes are parallel since only denatured protein is present. The slope gives the intrinsic temperature dependence of rotation of unfolded protein. Rotations in solutions having different refractive indices are not directly comparable[8] and for this reason the upper slopes do not lie on a common line. For ribonuclease there was clear-cut evidence for this interpretation as the upper slopes did fall on a common line after refractive index corrections were applied. The data presented in Fig. 1 cannot be interpreted so easily, and we must argue in part by analogy with ribonuclease. No refractive index data are available for concentrated urea solutions at 365 mμ though it should be possible to make the necessary measurements in this laboratory in the near future. But even with the corrections it is clear that the upper slopes cannot all coincide. For example, if it had been measured, the upper slope in the 0 M solution could not coincide with that of the 8 M solution even after being corrected. The reason for this is that the corrections will reduce the rotations for the 8 M but not for the 0 M solution. However, judging from the corrections at 589, the upper slopes for the 8 and 10 M solutions will probably coincide after the refractive index corrections have been made. (It has been shown that these corrections are not strongly dependent on wavelength[8].) (d) Below the temperatures of the minima in the rotation-temperature curves an inverted transition is occurring; *i.e.*, some protein is being denatured as the temperature is decreased. (e) At temperature below the minima in Fig. 1 the slope is greater for the 10 M solutions for two reasons. First in 10 M urea more of the unfolded lysozyme is present at low temperatures than in the 8 M solution as judged from the larger rotation. Since the unfolded material has a larger intrinsic temperature dependence of rotation, a steeper slope is observed for the 10 M solution. Secondly, the inverted transition will lead to a greater decrease in rotation as the temperature is increased.

The arguments for these interpretations are given in greater detail in the previous paper on ribonuclease.

On comparing the data of Fig. 1 with the comparable data for ribonuclease it is apparent that lysozyme is considerably more stable under the conditions of the experiments. For example, judging from the change in rotation no configurational change has occurred in lysozyme on heating at 70°. In ribonuclease, however, the protein has gone through the entire transition by 72° *i.e.*, the maximum in the rotation-temperature curve is observed at this temperature. In 8 M urea ribonuclease is completely unfolded even at 5° while lysozyme only begins to unfold at 30°.

Thermodynamics

Although the rotation-temperature data are not completely reversible, it is possible to make use of the VAN 'T HOFF relationship to estimate the change in enthalpy on unfolding[11]. However, as mentioned in the RESULTS the rotation-temperature data for lysozyme in the absence of urea are changing markedly with time. For this reason and because it was not possible to satisfactorily complete the transition, the calculations have been made for the 8 M urea solution only. Plotting the logarithm of the equilibrium constant *versus* the reciprocal of the absolute temperature gives a reasonably straight line with a slope corresponding to 69 kcal. If a transition temperature is defined as that temperature at which the equilibrium constant is unity, a value of 47° is obtained and the entropy of unfolding at this temperature is 215 eu. It would be desirable to learn what the change in enthalpy is in the absence of urea. This would permit a comparison of the relative numbers of hydrogen bonds broken during the transition for the ribonuclease and lysozyme molecules assuming a negligible difference in side chain effects.

There has been only one comparable measurement reported to this writer's knowledge. HAMAGUCHI[9] has measured the temperature dependence of viscosity of lysozyme in 8 M urea and used a two state model to determine the enthalpy change. He reports a value of approx. 5 kcal and argues that this unusually small value implies a small structural change in the lysozyme on unfolding. However, it appears that an error was made in his calculations since HAMAGUCHI's own data leads to a value of 52 kcal and a transition temperature of 41°. Whether this difference between HAMAGUCHI's data and ours reflects a real difference in the lysozyme samples used or in the experimental procedure is not clear at present.

Recently a number of papers have been published discussing the statistical mechanics of the phase transition occurring in polypeptides[10–15]. In one of these **Peller** has shown that

$$T_t^\circ - T_t \approx \frac{R(T_0)^2}{\Delta h} 2KC$$

where T_t° is the transition temperature in the absence of denaturant, T_t is the transition when the denaturant is at concentration C. K is the average equilibrium constant for denaturant binding to the sites exposed after the configurational transition and Δh is the heat of binding for the same process. Since Δh is known to be small (approx. — 2 kcal) the temperature dependence of K may be neglected as a first approximation. Using reasonable values **Peller** has estimated a value of — 4.44 for the coefficient of C. The data for lysozyme and ribonuclease are in remarkably good agreement with this relationship as can be seen from Fig. 5. If the data for lysozyme are extrapolated to 0 M the transition temperature is found to be 78°. This extrapolation was made before any experiments had been successfully completed in the absence of urea and the prediction provided an impetus for completing such an experiment. As mentioned in the RESULTS it was not possible to very accurately measure the transition temperature but it is probably within one degree of the predicted value. Thus the transition temperature for lysozyme varies linearly with the urea concentration over the entire experimental range of 0 to 10 M urea. This agreement is additional evidence for the applicability of the very simple model used for the configurational transitions of proteins. The slope for the lysozyme data is -3.3 and that for the ribonuclease is -4.5. These are of the expected order of magnitude.

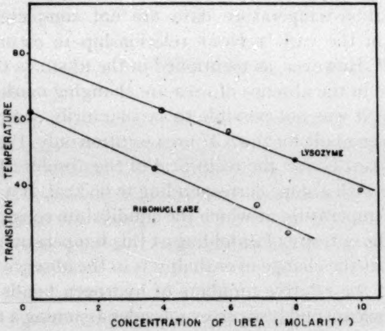


Fig. 5. Dependence of transition temperature of lysozyme and ribonuclease on concentration of urea.

However, if we assume common values for K and Δh for all proteins the slope for the lysozyme should be somewhat greater since its transition temperature in the absence of urea is greater. This suggests that such an assumption is incorrect, which is not surprising.

Peller has also pointed out the interesting similarity between this equation for the transition point depression and the usual expression for the freezing point depression of a solvent by the addition of solute. The folded protein would correspond to microcrystals of pure solvent in the solid phase and the unfolded protein to protein in the liquid phase. This would agree with KAUZMANN's suggestion[16] that protein configurational transitions are similar to the melting of ordinary crystals but with a relatively broad "melting point", because the crystal size is so small.

Fig. 5 also shows quite clearly that the 5 M solution of Fig. 2 is quite different in its behavior from that expected for native lysozyme. While the $a(T)$ curve was not carried to completion a reasonable estimate of the midpoint of the transition would be 50°, but Fig. 5 shows that a value of 59° would be expected. One simple interpretation of this behavior is that the refolding which takes place on removing the urea does not occur in the most efficient manner; *i.e.*, the manner which would put it into its state of lowest free energy. An interesting corollary of this is that if one were to very slowly remove the urea the final state might more closely approximate the native protein. Similarly, cooling a protein slowly might help to return it to a more native-like state since this would increase the rate of attainment of equilibrium unless of course irreversible effects take place during the time it is at the elevated temperature.

Kinetics

The rapid reversal experiments described are quite interesting and in part qualitatively different from earlier reports on the reversal of protein denaturation. The temporary entrapment of the protein in the high temperature, unfolded form is similar to a supercooling effect in an ordinary phase transition. We have attempted to evaluate the activation for this reversal process but the data are not accurate enough to give reliable values. It is clearly very large (probably greater than 100 kcal) and consequently any small error in the sample temperature on cooling will give rise to marked changes in the velocity of refolding. (As mentioned earlier, there may have been small errors in the temperatures used for the activation energy calculations. These can be eliminated by the use of the titanium polarimeter tube and the direct measurement of temperature with a thermistor probe.)

Only one experiment is shown in Fig. 3 in which mercuric ions were used to partially block the reversal. Such experiments might indicate what groups participate in the refolding. For example it is thought that mercuric ions may bind weakly to histidine side chains in papaya lysozyme. It may also be possible to more directly get at this question of why agents such as mercuric ions block the reversal by following the reactions with difference spectra measurements. Some preliminary measurements (but in the absence of blocking agents) have been made of the difference spectrum between an unheated sample of lysozyme in 10 M urea and a sample temporarily trapped in the unfolded state. Two distinct peaks located near 292 and 237 mμ have been observed which decrease with time. These promising experiments are being continued.

ACKNOWLEDGEMENTS

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MECHANISM OF INHIBITION OF D-AMINO ACID OXIDASE

IV. INHIBITORY ACTION OF CHLORPROMAZINE

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SUMMARY

1. The interaction of chlorpromazine and FAD was studied in aqueous solution and in a reaction mixture containing D-amino acid oxidase. The formation of a complex between chlorpromazine and FAD and the competition of these compounds for the apo-enzyme were demonstrated.

2. The formation of complexes of chlorpromazine and flavins was demonstrated by means of spectrophotometry and fluorimetry. The dissociation constant of the complex of chlorpromazine with FAD at pH 7.0 and 20°, measured by fluorimetry, appeared to be $1.0 \cdot 10^{-3}\ M$. The same dissociation constant was found for the chlorpromazine complexes with riboflavin, FMN or riboflavin 5′-monosulfate.

3. The competition of chlorpromazine with FAD for the apo-enzyme was demonstrated by means of kinetic analysis. The dissociation constant of the complex of chlorpromazine with the apo-enzyme at pH 8.3 was calculated to be $2.3 \cdot 10^{-5}\ M$.

4. The binding site of the apo-enzyme with chlorpromazine was determined by using riboflavin 5′-monosulfate and adenosine 5′-monosulfate as indicators. The results showed that chlorpromazine competes with adenosine 5′-monosulfate for the apo-enzyme, which means that chlorpromazine binds at the same site as the AMP moiety of FAD.

Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin monophosphate; AMP, adenosine monophosphate; FMS, riboflavin 5′-monosulfate; AMS, adenosine 5′-monosulfate.

INTRODUCTION

In our series of reports[1-3] on the mechanism of the inhibition of D-amino acid oxidase, the complex formation of a certain substance with FAD which resulted in the inhibition of the oxidase was demonstrated; it was also shown that this substance competes with FAD.

As previously reported, chlorpromazine competes with FAD in a reaction mixture containing D-amino acid apo-oxidase[4], and also forms a complex with FAD in aqueous solutions[5]. This complicated inhibitory action was of interest in the study of the mechanism of inhibition of D-amino acid oxidase.

However, the inhibitory action of chlorpromazine on flavin enzyme had been assumed to have some connection with the pharmacological effects of chlorpromazine in the brain[4]. Moreover, Löw[6] reported recently that this action may be related to the uncoupling action of chlorpromazine in the oxidative phosphorylation of rat liver mitochondria.

Consequently, it was considered that the elucidation of the mechanism of the inhibition of flavin enzyme by chlorpromazine would provide valuable suggestions for the study of the actions of chlorpromazine in the living body.

Hence the precise mechanism of the inhibitory action of chlorpromazine was studied from this point of view.

MATERIALS

D-amino acid oxidase apo-protein, free from FAD, was prepared by the method of NEGELEIN AND BRÖMEL[7]. FAD was prepared by the method of YAGI *et al.*[8]. Chlorpromazine–HCl, N-(3′-dimethylaminopropyl)-3-chlorophenothiazine hydrochloride, was supplied by Rhône-Poulenc-Specia. FMS and AMS were synthesized by the method of TAKAHASHI, YAGI AND EGAMI[9] and TAKAHASHI AND EGAMI[10], respectively.

METHOD

Investigation of the complex formation of chlorpromazine with FAD

The complex formation of chlorpromazine with FAD was examined by spectrophotometry and fluorimetry.

The shift of the absorption spectrum of FAD by chlorpromazine was measured by a Beckman type DU spectrophotometer. The quenching of the fluorescence of an aqueous solution of FAD by chlorpromazine was analysed by using a fluorimeter designed by YAGI *et al.*[11]. Assuming that the reaction of chlorpromazine and FAD is bimolecular and that the complex is non-fluorescent, the relation between the fluorescence intensity of FAD and the concentration of chlorpromazine (C) can be shown to be

$$\frac{f}{f'} = 1 + \frac{C}{K} \qquad (1)$$

where f and f' are the fluorescence intensities of FAD in the absence and presence of chlorpromazine respectively and K is the dissociation constant of chlorpromazine from its complex with FAD. By plotting f/f' against C, a straight line with the intercept 1 can be obtained, and K can be calculated from the slope of this line.

Spectrophotometric and fluorimetric analyses were also applied to examine the interaction between chlorpromazine and riboflavin, FMN or FMS.

Kinetic study of the competition of chlorpromazine with FAD

The oxidase activity was measured as described previously[1-3]. The dissociation constant of FAD with the apo-protein (K_f) was calculated to be $1.1 \cdot 10^{-7}\,M$ using the equation of MICHAELIS–MENTEN.

Assuming that chlorpromazine forms a complex with the apo-protein in competition with FAD, the enzyme activity can be represented as,

$$v = \frac{Vf}{K_f(1 + i/K_{ip}) + f} \tag{2}$$

where v is the reaction velocity in the presence of concentrations of f and i FAD and chlorpromazine respectively, V is the maximum velocity obtained in the presence of a large excess of FAD, and K_{ip} is the dissociation constant of chlorpromazine with the apo-protein in competition with FAD.

Using the LINEWEAVER–BURK form of eqn. (2), K_{ip} can be calculated from the slope of the straight line obtained by plotting $1/v$ against $1/f$ (Method I).

From the eqn. of MICHAELIS–MENTEN and eqn. (2), the following can be deduced,

$$\frac{v_0}{v} = 1 + \left\{1 - \frac{v_0}{V}\right\} \frac{i}{K_{ip}} \tag{3}$$

where v_0 and v are the reaction velocities in the absence and presence of chlorpromazine respectively. If the values of v_0/v plotted against i give a straight line with an intercept of 1, the inhibitory action of chlorpromazine can be considered to have only one mechanism as previously reported[2]. K_{ip} can also be calculated from the slope of the straight line thus obtained (Method II).

The elucidation of the binding site of the apo-protein with chlorpromazine was carried out by the method previously reported using FMS and AMS as indicators[3]. Assuming that a substance combines with the protein in competition with both FAD and one indicator, v_0/v can be shown as,

$$\frac{v_0}{v} = 1 + \left\{1 - \frac{v_0}{V}\right\} \left\{\frac{i_1}{K_1} + \frac{i_2}{K_2}\right\} \tag{4}$$

where K_1 is the dissociation constant of the complex of the substance with the protein, and K_2 is that of the complex of the indicator with the protein. i_1 and i_2 are the concentrations of the unknown substance and the indicator respectively. When the substance combines with the protein in competition with FAD but not with the indicator, v_0/v can be shown to be

$$\frac{v_0}{v} = 1 + \left\{1 - \frac{v_0}{V}\right\} \left\{\frac{i_1}{K_1} + \frac{i_2}{K_2} + \frac{i_1 i_2}{K_1 K_3}\right\}$$

or,

$$\frac{v_0}{v} = 1 + \left\{1 - \frac{v_0}{V}\right\} \left\{\frac{i_1}{K_1} + \frac{i_2}{K_2} + \frac{i_1 i_2}{K_2 K_4}\right\} \tag{5}$$

where K_3 is the dissociation constant of (apo-protein-i_1) + $i_2 \rightleftharpoons$ (apo-protein-i_1-i_2) and K_4 is that of (apo-protein-i_2) + $i_1 \rightleftharpoons$ (apo-protein-i_1-i_2). When the position of the binding site of the protein with one inhibitor does not influence the binding site of the protein with the other inhibitor, *i.e.*, no interaction takes place between the two binding sites of the protein, $K_1 = K_4$ and $K_2 = K_3$, and v_0/v can be shown to be

$$\frac{v_0}{v} = 1 + \left\{1 - \frac{v_0}{V}\right\} \left\{\frac{i_1}{K_1} + \frac{i_2}{K_2} + \frac{i_1 i_2}{K_1 K_2}\right\} \tag{6}$$

Plotting v_0/v against the concentrations of the inhibitors, a straight line can be obtained in the case of eqn. (4), and a curve in the case of eqn. (5) or (6). Thus it can be determined from the form of the curve whether the substance competes with the indicator or not, and, consequently, the part of the FAD which competes with the substance may be deduced (see previous report[3]).

Since, as mentioned before, chlorpromazine forms a complex with FMS, this complex formation should also be considered in the kinetic analysis shown above. The decrease of the effective concentrations of both chlorpromazine and FMS caused by their complex formation must be observed when the complex does not act as a competitive inhibitor.

In this case, the concentration of two inhibitors (i_1, i_2) in eqns. (4), (5) and (6) should be replaced by the concentration of the free inhibitors ($i_1 - x$), ($i_2 - x$), which can be approximately calculated from the following equation:

$$K = \frac{(i_1 - x)(i_2 - x)}{x} \tag{7}$$

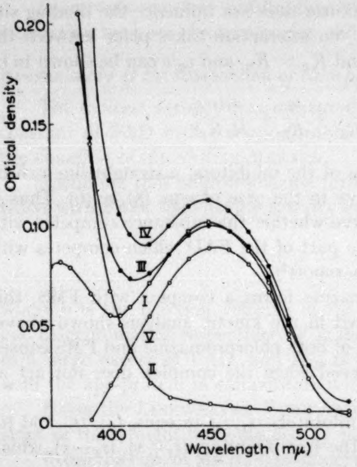
where K is the dissociation constant of the complex formed between two inhibitors in an aqueous solution, obtained by using eqn. (1). x is the concentration of the complex of chlorpromazine with FMS.

RESULTS

Complex formation of chlorpromazine with flavins

In the preliminary experiments, it was shown that the addition of a large excess of chlorpromazine to FAD solution (pH 6.5, 20°) changes the colour of the latter from yellow to brownish-yellow and also diminishes the fluorescence of the solution. So, it was expected that a change in the absorption spectrum of FAD would occur due to the formation of a complex with chlorpromazine. As shown in Fig. 1, the measured optical density of the chlorpromazine–FAD mixture in the visual wave-length region was considerably suppressed compared with its theoretical value (see curves III and IV). The peak in the difference absorption spectrum of FAD was shifted slightly to the longer wave-length region on the addition of chlorpromazine (see curves I and V). Similar changes were also observed in the cases of riboflavin, FMN and FMS.

The quenching of the fluorescence of FAD was also demonstrated by the addition of chlorpromazine. The plot of f/f' against the concentration of chlorpromazine gave a straight line with an intercept of 1, as shown in Fig. 2. From the slope of this line, K was calculated to be $1.0 \cdot 10^{-3}\,M$. The same dissociation constant was obtained for the complex of chlorpromazine with riboflavin, FMN or FMS using the same procedure.



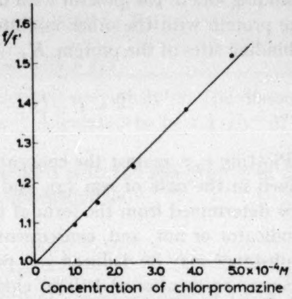


Fig. 2. Quenching action of chlorpromazine on the fluorescence of FAD: f and f' correspond to the fluorescence intensities of FAD ($1.0 \cdot 10^{-6}$ M) in the absence and presence of chlorpromazine in phosphate buffer (M/10, pH 7.0) at 20°.

Fig. 1. Spectra of FAD and chlorpromazine: I, FAD ($9.0 \cdot 10^{-6}$ M) in phosphate buffer (M/10, pH 6.5); II, chlorpromazine ($2.0 \cdot 10^{-3}$ M) in the same buffer; III, mixture of FAD ($9.0 \cdot 10^{-6}$ M) and chlorpromazine ($2.0 \cdot 10^{-3}$ M) in the same buffer; IV, theoretical spectrum of FAD + chlorpromazine; V, the difference spectrum of FAD (III–II).

Competitive inhibition of chlorpromazine with FAD

In the presence of $2.0 \cdot 10^{-5}$ M chlorpromazine in the enzyme reaction mixture, a plot of $1/v$ against $1/f$ gave a straight line with an intercept of $1/V$, as shown in Fig. 3 (Method I). From the slope of this line, K_{ip} was calculated to be $2.3 \cdot 10^{-5}$ M.

A plot of v_0/v against the concentration of chlorpromazine also gave a straight line with an intercept of 1, as shown by curve I in Fig. 4 (Method II). From the slope of this line, K_{ip} was also calculated to be $2.3 \cdot 10^{-5}$ M in good agreement with the value obtained by Method I.

Binding site of the apo-protein with chlorpromazine

Kinetic analysis of the binding site of the apo-protein with chlorpromazine was carried out by using FMS and AMS as the indicators. The plots of v_0/v against the concentrations of both chlorpromazine and AMS gave a straight line, as shown by curve II in Fig. 4. In the presence of both chlorpromazine and FMS, plots of v_0/v against the concentration of both inhibitors gave a second order curve, as illustrated by curve III in Fig. 4.

DISCUSSION

In the present study it was found that chlorpromazine inhibits D-amino acid oxidase in competition with FAD (dissociation constant, $2.3 \cdot 10^{-5}$ M) and at the same time forms a complex with flavin (dissociation constant, $1.0 \cdot 10^{-3}$ M).

In the previous papers[2,12], similar cases were found *i.e.*, *p*-aminosalicylic acid or *p*-aminophenol forms a complex with and, at the same time, competes with FAD

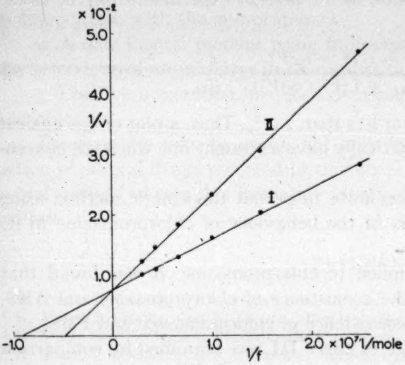


Fig. 3. Inhibitory action of chlorpromazine on D-amino acid oxidase in competition with FAD (Method I). Reaction mixture contained 15 μg of the oxidase protein, 0.1 M (final concn.) of DL-alanine and indicated concentration of FAD. After the temperature equilibrium had been reached, $2.0 \cdot 10^{-5}$ M (final concn.) of chlorpromazine or water was added to the main chamber from the side bulb. Curve I: in absence of chlorpromazine. Curve II: in presence of chlorpromazine.

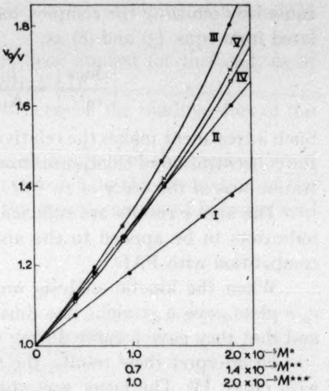


Fig. 4. Competitive inhibition of chlorpromazine with FAD (Method II) and analysis of the binding sites of the protein with chlorpromazine using kinetic indicators. v_0 and v represent the reaction velocities in the absence and presence of inhibitors. Reaction mixture contained 15 μg of the oxidase protein, 0.1 M (final concn.) of DL-alanine and $1.7 \cdot 10^{-7}$ M (final concn.) of FAD in the absence and presence of i concentration of inhibitors: * chlorpromazine concentration; ** FMS concentration; *** AMS concentration. Curve I: in the presence of chlorpromazine. Curve II: in the presence of chlorpromazine and AMS. Curve III: in the presence of chlorpromazine and FMS. Curves IV and V: theoretical values of v_0/v (see the text). V was 132 μl uptake of oxygen per 30 min and v_0 was 80 μl uptake of oxygen per 30 min.

for the protein in D-amino acid oxidase reaction mixture. In the case of p-aminosalicylic acid, the plot of v_0/v against the concentration of p-aminosalicylic acid gave a second order curve, which indicated that two different mechanisms were involved in the inhibitory action. In this case, v_0/v was expressed by the following equation[2, 12],

$$\frac{v_0}{v} = 1 + \left\{1 - \frac{v_0}{V}\right\} \left\{\left(\frac{1}{K_{ip}} + \frac{1}{K_{if}}\right)i + \frac{i^2}{K_{ip}K_{if}}\right\} \tag{8}$$

where K_{if} is the dissociation constant of the inhibitor–FAD complex in the oxidase reaction mixture. K_{if} and K_{ip} of p-aminosalicylic acid were reported as $7.5 \cdot 10^{-2}$ M and $1.6 \cdot 10^{-2}$ M respectively.

In the present study, however, a plot of v_0/v against the concentration of chlorpromazine practically gave a straight line, which shows that a single mechanism dominates the inhibitory action.

This phenomena can be attributed to the difference between the order of the dissociation constant of the chlorpromazine–FAD complex and that of the concentration of chlorpromazine. Considering that the former is 100 times larger than the latter, the complex formation of chlorpromazine with FAD is negligible compared with the competition of chlorpromazine with FAD. Actually, the theoretical error caused by omitting the complex formation in the present experiment may be calculated from eqns. (3) and (8) as,

$$\left|\frac{\Delta v_0/v}{v_0/v}\right| = \frac{i\,(K_{ip} + i)\,(1 - v_0/V)}{K_{if}\{K_{ip} + i\,(1 - v_0/V)\}} \leq \frac{i}{K_{if}} \tag{9}$$

Such a treatment makes the relative error less than 2.0 %. Thus, a plot of v_0/v against the concentration of chlorpromazine practically gave a straight line when the concentration was of the order of 10^{-5} *M*.

The above results are sufficiently accurate to permit the kinetic method using indicators to be applied to the analysis of the behaviour of chlorpromazine in its competition with FAD.

When the kinetic analysis was applied to chlorpromazine, it was found that v_0/v plots gave a straight line during the coexistence of chlorpromazine and AMS, and that they gave a curve during the coexistence of chlorpromazine and FMS.

To interpret these results, the nature of curve III was examined by comparison with curve IV. The latter was obtained from the theoretical values calculated as follows: assuming that no interaction takes place between the two binding sites of the apo-protein, eqn. (6) was adopted. In this case, the complex formation of chlorpromazine with FMS has to be considered. Then, assuming that the complex of the two inhibitors can not act as an inhibitor, the concentration of free inhibitors was calculated approximately from eqn. (7). Substituting the concentration of free inhibitors calculated from eqn. (7) for i_1 and i_2 in eqn. (6), the theoretical values of v_0/v were calculated as shown by curve IV in Fig. 4.

The fact that curve III is more convex against the abscissa than curve IV shows that there exists an interaction between the two binding sites of the apo-protein with chlorpromazine and FMS.

Curve III was examined further by comparing it with another theoretical curve, curve V, which was deduced using the assumption that neither interaction between the two binding sites of the apo-protein nor complex formation of chlorpromazine with FMS occurred. Curve III was found to be even more convex than curve V.

The results mentioned above were interpreted as follows: Chlorpromazine combines with apo-protein in competition with the AMP part of FAD, and interaction takes place between the binding sites of the apo-protein with chlorpromazine and with FMS.

Complex formation of chlorpromazine with flavins is considered to be significant *in vivo*. The fact that the modification of the electroencephalogram by the administration of chlorpromazine is reversed by FAD may be partly explained by the complex formation of these two compounds *in vivo*[13].

Recently, Löw reported that the uncoupling action of chlorpromazine on oxidative phosphorylation is connected with FAD or FMN. The present results concerning the complex formation of chlorpromazine with flavins may furnish actual evidence for the participation of FAD in oxidative phosphorylation reported by GRABE[14]. It is noteworthy that substances known to be uncouplers of oxidative phosphorylation, namely, 2,4-dinitrophenol[2], chlortetracycline[1] and chlorpromazine[4, 5] are all equally found to form complexes with flavins.

In concluding this series of reports, the following results emphasized throughout the four papers may be mentioned:

1. Some substances can inhibit D-amino acid oxidase by combining with the FAD in competition with the apo-protein.

2. A new kinetic method using indicators has been applied for the analysis of the behaviour of an inhibitor in its competition with FAD.

3. Positive interaction has been demonstrated between the binding sites of the apo-protein.

These results may be useful not only for the elucidation of the mechanism of action of several drugs reported in this series, but also for the demonstration of the actual method of complex formation involved in the enzyme action of D-amino acid oxidase.

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A RAPID ASSAY METHOD FOR TRITIUM IN BACTERIAL CELLS

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SUMMARY

1. The use of a simple bacterial filtration technique involving collodion membranes has permitted a precise means of assaying tritiated compounds. Radioassays at infinite thickness were readily converted to relative total count values. It is suggested that the method has general applicability.

2. Bacterial cultures rapidly converted exogenous tritiated thymidine to thymine. [^{3}H]diaminopimelic acid served as a more stable biochemical precursor, enabling accurate studies on the incorporation of tritium into bacterial cells.

Abbreviation: TCA, trichloroacetic acid.

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INTRODUCTION

The ready availability of relatively inexpensive tritium-labeled biochemicals has made their use particularly attractive for investigation of metabolic processes. However, the difficulties associated with detection and measurement of radioactivity from such a weak β-emitter have discouraged many workers. Unquestionably, the most sensitive and precise method for measurement of this isotope is the internal liquid scintillation method[1]. This method requires the availability of expensive equipment beyond the reach of most laboratories, and consequently several investigators have applied the method of conventional windowless gas-flow counting[2–4]. Reproducibility is not very satisfactory because of the difficulty of obtaining reliable geometry of distribution. A simple procedure was sought, therefore, for the assay of tritium that would be particularly suitable for existing windowless gas-flow equipment.

The membrane filtration procedure has been employed in this laboratory for the rapid and quantitative recovery of bacterial[5] and tumor[6] cells for the radioassay of ^{14}C. In this technique, cells were removed from their suspensions and were spread quite evenly on the filters. This method has now been applied to the assay of ^{3}H in bacterial cells. Infinite thickness levels were reached readily, and the uniform geometry allowed reproducible count rates. From the specific activity values derived by this technique, highly reproducible values for relative total activity were obtained with a minimum of manipulation or expense.

METHODS

Bacillus cereus, strain 569 H, was grown in a casein hydrolysate-salts medium, as described[5]. Growth was assayed by turbidity measurements at 540 mμ in a Beckman spectrophotometer, Model DU. 2-ml samples of the culture to which a radioactive compound had been added (approx. 0.15 μC/ml of medium) were removed for turbidimetric assay and immediately afterward mixed with an equal volume of 10% TCA. The acid-treated cells were filtered through collodion membrane filters (Schleicher and Schuell Co., Keene, New Hampshire, U.S.A.), and washed with two 5-ml portions of 1% TCA. The filters were mounted in aluminum planchettes with rubber cement and were assayed for radioactivity in a windowless gas-flow counter using natural gas for counting. The membrane method has been described by BRITTEN *et al.*[7] of the Carnegie Institution of Washington, Washington, D.C.

Bacteria washed with saline were fractionated by extraction with cold TCA, aqueous ethanol, ethanol–ether and hot TCA, as described[8]. The residue, termed "total protein fraction" was hydrolyzed with 6 *N* HCl in a pressure cooker at 8 lbs/in^2 (112°) for 3 h.

[^{3}H]thymidine (360 μC/μmole) was obtained from Schwarz Laboratories, Mount Vernon, N.Y., and [^{3}H]diaminopimelic acid was kindly furnished by Dr. J. L. STROMINGER, Department of Pharmacology, Washington University School of Medicine, St. Louis, Mo. (U.S.A.). [Me-^{14}C]thymidine (14 μC/μmole) was obtained from the Cancer Chemotherapy National Service Center, National Institutes of Health, Bethesda, Md. (U.S.A.).

RESULTS

Experiments with [^{3}H]thymidine

Thymidine was used in these experiments because the compound was known to be utilized for DNA synthesis by *B. cereus*[9]. Varying quantities of a cell suspension of *Bacillus cereus* which had been grown in the presence of [^{3}H]thymidine were filtered by the usual procedure. A self-absorption curve (Fig. 1) indicated that constant count rates were obtained, once infinite thickness levels of radioactive cells had been plated. In these experiments, maximal radioactivity assays were observed after 2.5 ml of bacterial suspension had been filtered, corresponding to 0.25 mg dry wt./cm^2. The absolute area density of ^{3}H labeled compounds for infinite thickness has been shown to be somewhat variable by HANNGREN[4], depending upon the nature of the compound and the means of plating it. Since it is difficult to reliably correct measurements of ^{3}H radioactivity for self-absorption, one must therefore depend upon specific activity measurements, which are obtained simply and precisely by the technique described.

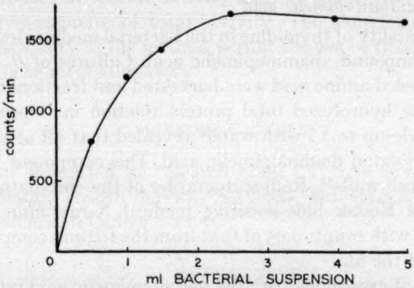

Fig. 1. Radioactivity, in counts/min, on membranes after filtration of varying volumes of suspensions of *B. cereus* grown in the presence of [^{3}H]thymidine. At OD_{540} of 0.4, 1 ml of suspension contained approx. $2 \cdot 10^8$ cells or 0.3 mg dry wt. Area of grid, 2.85 cm^2.

Kinetic experiments on cellular incorporation of exogenous [^{3}H]thymidine revealed that the rapid bacterial uptake of radioactivity ceased after a short period of incubation, and specific activities of the isolated cells began to decrease with time. Although the growth medium, separated from the cells by centrifugation, was still highly radioactive, and newly inoculated cells proliferated in a normal fashion in this medium, the cells produced were essentially devoid of radioactivity. Each additional supplementation of [^{3}H]thymidine resulted in a period of renewed incorporation of label, and it was concluded that in the presence of the growing cells, the isotopic compound was rapidly transformed into a less readily incorporated product. Addition of large quantities of carrier thymidine with the isotope slowed the rate of destruction, and a longer period of isotopic uptake was observed.

Paper chromatography (70 % aqueous isopropanol saturated with NH_3)[10] of the bacterial medium after the uptake of [^{3}H]thymidine had terminated, revealed the presence of a metabolite which corresponded in its u.v. absorption characteristics at pH 7 and pH 13 and in R_F value to those of thymine. The slower rate of uptake of the free base, in comparison with that of the deoxyriboside, would explain the drop in specific activities observed if, after the decomposition of exogenous thymidine, thymine of DNA were synthesized from a source other than the labeled free base present, such as by methylation of deoxyuridine[11]. The instability of thymidine in the presence of bacteria was also observed with a wild strain of *E. coli* (ATCC 11303), as well as a thymine requiring mutant of *E. coli*[12]. Prusoff[13] has reported a similar cleavage by *Streptococcus faecalis*.

Parallel experiments with [Me-^{14}C]thymidine confirmed that the incorporation of the compound into bacterial cells almost ceased after a period of incubation.

Experiments with [^{3}H]diaminopimelic acid

The biological instability of thymidine in the bacterial medium led to examination of another tritiated compound, diaminopimelic acid. Cultures of *B. cereus* grown in the presence of this labeled amino acid were harvested and fractionated as described. Chromatography of the hydrolyzed total protein fraction in isopropanol (680 ml)–conc. HCl (176 ml) made up to 1 l with water[8] revealed that all of the radioactivity was recovered in the isolated diaminopimelic acid. This compound has been shown to be a constituent of cell walls[14]. Radioautography of the chromatograms led to no darkening of Eastman Kodak blue-sensitive medical X-ray film, whereas radiocarbon chromatograms with counts 0.05 of that from the tritium compounds produced noticeable darkening of the film.

The incorporation of exogenous tritiated diaminopimelic acid into exponentially growing cells of *B. cereus* was followed by periodic sampling of the culture using the membrane technique. The turbidity of the culture was recorded simultaneously. The results, shown in Fig. 2, are represented in terms of the incorporation of radioactivity as a function of increase in turbidity. Relative total radioactivity has been computed by multiplying the specific activity values obtained from direct count of the filters by the turbidity of the bacterial suspension, the latter being a function of cell mass. As apparent from Fig. 2, the incorporation of the isotopic precursor was directly related to the increase in cell mass, thus validating the present procedure with tritium.

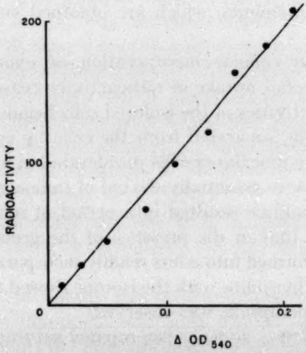

Fig. 2. Incorporation of radioactive diaminopimelic acid into *B. cereus* as a function of bacterial growth. Total activity units computed from counts and mass of cells for the sample filtered. Growth (ΔOD_{540}) measured turbidimetrically at 540 mμ.

DISCUSSION

The method described here may be applied to the assay of tritium in any system of an insoluble, filterable precipitate which contains the radioactivity of the material being assayed. It is ideally suited for assay of bacterial cells or of dispersed animal cells such as ascites tumor cells or blood cells. It is also applicable to the measurement of an insoluble intracellular component such as nuclear DNA. It must be born in mind that the measurement of specific activity alone is not limiting if an independent measure of concentration, such as turbidimetric measurements or cell counts are available. The product of specific activity and the concentration measurement gives a suitable relative estimate of total activity. The simplicity, reproducibility, and inexpensive applicability of the method permit the use of tritium labeled compounds in almost any biochemical laboratory.

ACKNOWLEDGEMENTS

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SÉPARATION ET CARACTÉRISATION DU L(+)-γ-(*p*-HYDROXY)ANILIDE DE L'ACIDE GLUTAMIQUE À PARTIR DE *AGARICUS HORTENSIS*

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SUMMARY

*Separation and characterisation of the γ-(*p*-hydroxy)anilide of glutamic acid from* A. hortensis

The γ (*p*-hydroxy)anilide of glutamic acid has been isolated from the mushroom *Agaricus hortensis* by chromatography on activated charcoal and column of Dowex 1 × 8.

The new compound was treated with 6 *N* hydrochloric acid and the products of hydrolysis have been identified as glutamic acid and *p*-aminophenol, the nature of these two substances being confirmed by comparison with synthetic materials. The compounds synthesized from *p*-aminophenol and pyrrolid-2-one-5-carboxylic acid appeared to be identical with the natural anilide.

INTRODUCTION

L'étude, par chromatographie bidimensionnelle et chromatoélectrorhéophorèse sur papier, de la fraction soluble dans l'alcool du champignon *Agaricus hortensis* a montré la présence dans celle-ci d'un nouveau dérivé de l'acide glutamique.

Dans une communication antérieure[1], nous avons décrit l'isolement de ce composé par adsorption sur charbon activé, élution, extraction de la phénylalanine, de la tyrosine et des autres impuretés aromatiques par l'eau glacée. Le résidu constitué, principalement par la substance à isoler a été recristallisé dans l'eau. Nous donnons également dans cette publication un tableau des valeurs des R_F de cette substance dans différents solvants. Cette technique ne donnant de bons résultats que pour des champignons absolument frais, une méthode de séparation utilisant les résines échangeuses d'ions a été mise au point. La substance donne une coloration pourpre avec la ninhydrine et rouge avec l'acide sulfanilique ou la *p*-bromoaniline, tous deux diazotés. Sur papier, la substance donne aussi une coloration jaune quand on la traite par une solution de nitrite de sodium à 10% additionnée de 2% d'acide acétique.

La Fig. 1 représente un chromatogramme bidimensionnel de l'extrait alcoolique de *Agaricus hortensis* réalisé en utilisant le *n*-butanol–acide formique–eau (75:15:10) et le phénol saturé par un tampon phosphate–acide citrique à pH = 4.2; la substance se trouve entre l'alanine et la proline, à droite de l'acide γ-aminobutyrique; si on

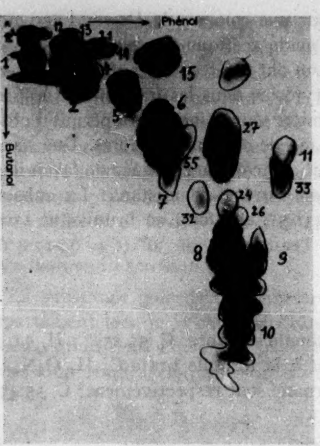


Fig. 1. Chromatogramme bidimensionnel de l'extrait d'*Agaricus hortensis* 1, acide aspartique; 2, acide glutamique; 3, sérine; 4, glycocolle; 5, thréonine; 6, alanine; 7, tyrosine; 8, valine; 9, phénylalanine; 10, leucines; 11, proline; 12, 12bis; 13, 14, acides aminés basiques; 15, glutamine; 21, asparagine; 24, tryptophane; 26, méthionine; 27, dérivés de l'acide glutamique; 32, acide α-aminobutyrique; 55, acide γ-aminobutyrique.

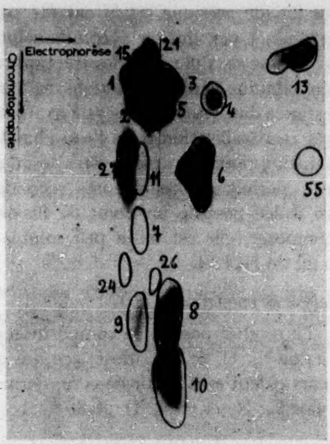


Fig. 2. Chromatoélectrorhéophorèse de l'extrait d'Agaricus hortensis Numérotation comme Fig. 1.

utilise le phénol ammoniacal, les spots de la substance et le l'acide γ-aminobutyrique sont confondus. La chromatoélectrorhéophorèse sur papier a été effectuée avec le solvant au butanol dans le sens de la chromatographie et avec une solution d'acide acétique N pour l'électrophorèse; dans ce cas (Fig. 2) la substance se trouve au dessus de la tyrosine, nettement séparée de l'acide γ-aminobutyrique qui migre à l'électrorhéophorèse comme les acides aminés basiques.

La substance est présente en quantité importante dans le champignon et se retrouve dans un très grand nombre de champignons du genre *Agaricus*, elle semble donc, du point de vue systématique, caractéristique de ce genre[2].

Isolement de la substance

Les champignons (2 kg) ont été pulvérisés dans un Waring blendor en présence de 8 l d'éthanol 95 % de façon à obtenir une concentration approximative de 80 % en éthanol dans l'extrait filtré. Après concentration par distillation sous pression réduite, l'extrait a été évaporé à sec. La substance, la phénylalanine et les autres impuretés aromatiques ont été adsorbées sur du charbon traité par l'acide acétique selon la méthode de PARTRIDGE[3], puis éluées par de l'acide acétique à 25 % contenant 5 % de phénol; l'éluat obtenu a été agité avec de l'éther pour éliminer le phénol, concentré par distillation sous pression réduite et évaporé à sec. La fraction aromatique a été ensuite dissoute dans l'eau et passée sur une colonne d'amberlite IR-120 forme H^+ et éluée par NH_3 *N*. L'éluat, évaporé à sec, a été dissous dans 100 ml d'acide acétique 0.5 *N*. La phénylalanine, la tyrosine et la nouvelle substance ont été séparées sur une colonne de Dowex 1 × 8 forme acétate: 50 ml de la solution dans l'acide acétique 0.5 N ont été passés sur une colonne de Dowex I (60 mm × 500 mm) et par élution avec une solution d'acide acétique de même concentration à la vitesse de 138 ml/h, des fractions de 45 ml ont été recueillies. On a retrouvé la phénylalanine dans les fractions 10 à 22, la tyrosine dans les fractions 28 à 36 et la substance dans les fractions 71 à 91. Cette première séparation nous a permis d'obtenir après recristallisation dans l'eau chaude 140 mg de cristaux incolores. Une seconde séparation portant sur les autres 50 ml de solution acétique et menée de façon identique a permis d'obtenir après recristallisation 110 mg de cristaux. La substance ainsi isolée possède un point de fusion de 231.5°, elle fond en brunissant sans se décomposer, elle est assez peu soluble dans l'eau; $\alpha_D^{20} = +30°$ (c = 0.217 g dans 100 ml de HCl N).

Analyse élémentaire

Le produit possède la composition centésimale suivante: C, 55.25 %; H, 5.94 %; N, 11.62 %; O, 27.3 % (dosé); ce qui conduit à la formule brute $C_{11}H_{14}O_4N_2$. Les valeurs calculées à partir de cette dernière formule sont respectivement: C, 55.45 %; H, 5.92 %; N, 11.76 %; O, 26.86 %.

Détermination de l'équivalent gramme

(a) Titration par l'acide perchlorique en milieu anhydre (acide acétique glacial): 12.27 mg de substance ont été titrés par 0.56 ml de $HClO_4$ 0.1 N, ce qui correspond à une valeur de 219 pour l'équivalent gramme. (b) Titration par le formaldéhyde: cette méthode donne un équivalent gramme de 230. (c) Titration par la méthode de TAGUE[4]: la titration par la soude 0.1 N permet de calculer un équivalent gramme de 219; signalons que, dans ces conditions, la tyrosine réagit non seulement par sa fonction acide mais aussi par sa fonction phénol.

Détermination du poids moléculaire

La détermination du poids moléculaire par la méthode de RAST modifiée par WENDT[5] donne une valeur de 236.

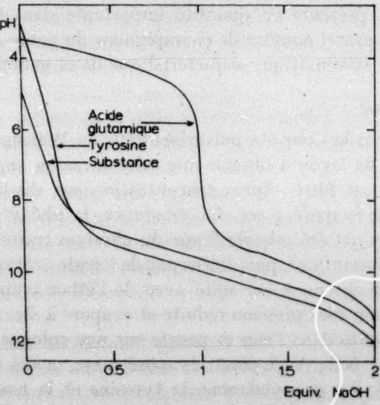


Fig. 3. Courbe de titration de la substance naturelle, de la tyrosine et de l'acide glutamique.

Le poids moléculaire trouvé est très voisin de 238, valeur calculée à partir de la formule $C_{11}H_{14}O_4N_2$. On peut donc adopter cette dernière comme formule moléculaire. La titrimétrie en milieu anhydre par $HClO_4$ donne un équivalent approché de 219 qui implique une fonction-NH_2 libre. L'équivalent de 230 obtenu par formoltitration implique la présence d'une fonction COOH libre. L'équivalent de 219 déterminé par la méthode de TAGUE prouve qu'il y a deux fonctions à caractère acide, la première acide carboxylique et la seconde acide très faible, vraisemblablement une fonction phénolique comme le suggère la comparaison des courbes de titration de la tyrosine et de la substance (Fig. 3).

Détermination du spectre u.v.

La substance possède un caractère aromatique marqué comme le montre son comportement lors de la chromatographie sur charbon activé et comme le confirme son absorption dans l'u.v. Son spectre u.v. est semblable à celui des autres acides aminés aromatiques, ce qui ressort du tableau suivant:

TABLEAU I

ABSORPTION DE DIFFÉRENTS ACIDES AMINÉS DANS L'U.V.

Acides aminés	*HCl 0.1 N*		*NaOH 0.1 N*	
	λ max (Å)	*ε max*	*λ max (Å)*	*ε max*
Phénylalanine	2575	195	2585	200
Tyrosine	2775	1500	2940	2550
Tryptophane	2780	5500	2810	5000
Substance	2460	11000	2630	9300

Le déplacement du $\lambda_{\max}$ vers les grandes longueurs d'onde quand on passe du milieu acide au milieu basique est de 165 Å pour la tyrosine et de 161 Å pour la substance étudiée. La valeur du $\varepsilon_{\max}$ est beaucoup plus grande dans le spectre u.v. de la substance que dans celui des autres acides aminés aromatiques.

Hydrolyse de la substance

Pour obtenir une hydrolyse complète, sans noircissement, il a suffi de chauffer à reflux la substance en solution dans HCl 6 *N* pendant 8 h. La solution a été ensuite évaporée dans un exsicateur en présence de KOH solide, le vide se faisant à la pompe à huile. On obtient de cette façon des cristaux incolores. Par chromatographie sur papier avec le solvant *n*-butanol–acide acétique–eau (4:1:5), on a pu mettre en évidence deux spots intenses. Le premier, coloré en jaune déjà lors du séchage du chromatogramme est devenu brun-rouge après révélation avec la ninhydrine; son R_F est de 0.4. Le second, violet après réaction avec la ninhydrine, est caractérisé par un R_F de 0.2. L'hydrolyse alcaline du composé par la baryte saturée pendant 1 h à 100° donne les mêmes résultats.

La migration des produits de l'hydrolyse a été étudiée par électrophorèse sur papier en présence d'un tampon phosphaté à pH = 5.6 pendant 1 h à 250 V. La substance correspondant au spot violet a migré comme l'acide glutamique et l'autre substance dans le sens des composés basiques.

Les deux produits d'hydrolyse ont été séparés sur une colonne de papier pressurisée à 0.35 kg/cm² dans un appareil Chromax L.K.B. à l'aide du solvant butanol–acide acétique–eau déjà cité. Ces produits ont également été séparés sur une colonne de Dowex 1 × 8 forme acétate à l'aide d'une solution d'acide acétique 0.5 *N*; la substance basique passant très rapidement à travers la colonne a été facilement séparée du composé acide fortement retenu.

Caractérisation de la substance basique

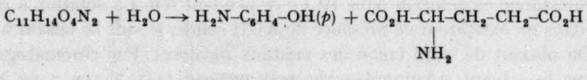
Celle-ci a été recristallisée deux fois dans l'éthanol absolu, ce qui a permis d'obtenir des cristaux fondant à 184°. Cette substance est à la fois soluble dans les acides et les bases mais ne décompose pas une solution de $NaHCO_3$; elle présente une absorption marquée dans l'u.v. (λ max = 286 mμ) et a donc un caractère aromatique; elle s'oxyde très rapidement en milieu basique.

L'analyse élémentaire donne les résultats suivants: C, 65.85 %; H, 6.36 %; N, 12.72 % (calculé pour C_6H_7ON: C, 66.03 %; H, 6.47 %; N, 12.84 %).

Ceci fait penser à un aminophénol. Le *p*-aminophénol fond à 184°. Le point de fusion mixte du mélange à 50 % de la substance à identifier et du *p*-aminophénol demeure à 184°. Comme les spectres i.r. des deux substances sont tout à fait comparables, on peut affirmer que le premier produit de l'hydrolyse est constitué par le *p*-aminophénol.

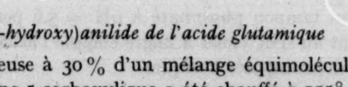
Caractérisation de la substance acide

Celle-ci a été recristallisée deux fois dans l'eau chaude, ce qui a permis d'obtenir des cristaux fondant à 213° avec décomposition. Par chromatographie sur papier à l'aide de différents solvants, il a été impossible de séparer cette substance de l'acide glutamique. L'analyse élémentaire des cristaux donne: C, 41.07 %; H, 6.27 %; N, 9.5 % (calculé pour $C_5H_9O_4N$: C, 40.81 %; H, 6.17 %; N, 9.52 %; poids moléculaire 147). Ce second produit d'hydrolyse titré par la méthode de TAGUE donne un équivalent gramme de 70 correspondant à un poids moléculaire de 140. Le point de fusion mixte du mélange de la substance et de l'acide glutamique provenant de diverses firmes commerciales se situe à 210°. Le spectre i.r. de la substance est identique à celui de l'acide glutamique et on peut affirmer que le second produit d'hydrolyse est constitué par de l'acide glutamique. La substance extraite de *Agaricus hortensis* a donc subi l'hydrolyse suivante:



$$C_{11}H_{14}O_4N_2 + H_2O \rightarrow H_2N\text{-}C_6H_4\text{-}OH(p) + CO_2H\text{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}\text{-}CH_2\text{-}CH_2\text{-}CO_2H$$

Le groupement NH_2 libre de $C_{11}H_{14}O_4N_2$ étant en α par rapport à la fonction CO_2H, la liaison amide doit se faire en γ et le nouvel acide aminé est le L (+)-γ-(*p*-hydroxy)-anilide de l'acide glutamique:



$$CO_2H\text{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}\text{-}CH_2\text{-}CH_2\text{-}C\begin{matrix}\nearrow O\\ \searrow NH\text{-}C_6H_4\text{-}OH(p)\end{matrix}$$

Synthèse du L (+)-γ-(p-*hydroxy*)*anilide de l'acide glutamique*

Une solution aqueuse à 30 % d'un mélange équimoléculaire de *p*-aminophénol et d'acide pyrrolid-2-one-5-carboxylique a été chauffé à 115° en tube scellé pendant

10 h. Après refroidissement et ouverture du tube, la solution a été évaporée sous vide. On a retrouvé dans le résidu, par chromatographie sur papier en présence de butanol saturé d'eau et d'acide formique, le γ-(*p*-hydroxy)anilide de l'acide glutamique ainsi qu'une partie de l'acide glutamique et du *p*-aminophénol non transformés. Le mélange a été séparé en ses composants par chromatographie sur une colonne de Dowex 1 × 8 et élution par l'acide acétique 0.5 *N*. Les fractions correspondant au nouveau dérivé de l'acide glutamique ont été concentrées et évaporées à sec. La substance recristallisée dans l'eau chaude fond à 228° et ne donne pas d'abaissement du point de fusion avec un échantillon du produit naturel. Elle contient 11.75 % d'azote (calculé pour $C_{11}H_{14}O_4N_2$: 11.76 %). Les spectres infrarouge et u.v. de la substance synthétique et du produit naturel sont identiques comme le montrent les Figs. 4 et 5.

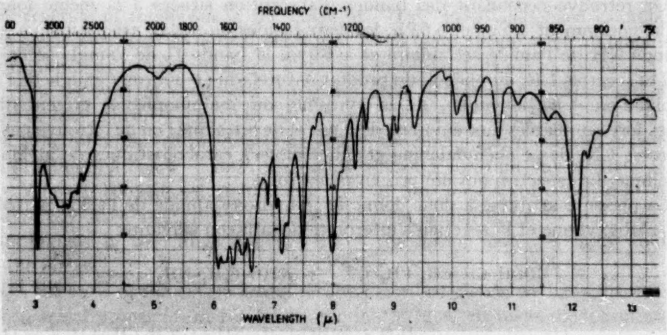


Fig. 4. Spectre infrarouge de la substance naturelle.

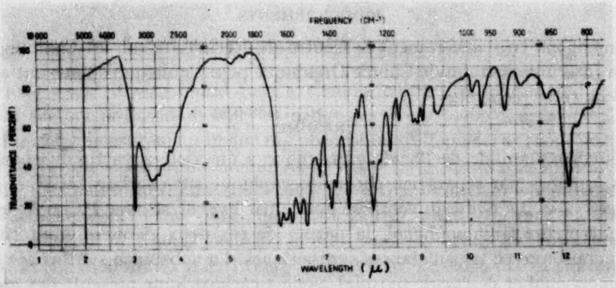


Fig. 5. Spectre infrarouge de la substance synthétique.

Synthèse du (p-*hydroxy*)*anilide de l'acide pyrrolid-2-one-5-carboxylique*

En vue d'améliorer le rendement de la synthèse, nous avons voulu modifier le mode opératoire; les nouvelles conditions expérimentales ne donnent pas le produit attendu mais bien le (*p*-hydroxy)anilide de l'acide pyrrolid-2-one-5-carboxylique.

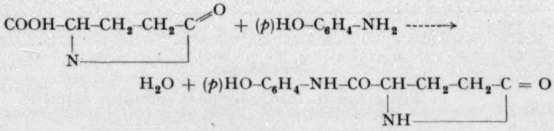
Un mélange à parties égales de *p*-aminophénol et d'acide pyrrolid-2-one-5-carboxylique a été chauffé à 190° sous atmosphère d'azote privé d'oxygène. Après fusion du mélange, on a laissé descendre la température à 160° et on l'y a maintenu pendant 20 min. Après refroidissement, la masse a été pulvérisée et lavée à l'éthanol chaud de façon à en assurer une décoloration complète. Le résidu a été extrait à l'eau chaude et par refroidissement de la solution aqueuse des cristaux blancs se sont formés. Après deux cristallisations dans l'eau, la substance présente un point de fusion constant de 285°. L'analyse élémentaire donne: C: 59,79%; H, 5,44%; N, 12.68%; calculé pour $C_{11}H_{12}O_3N_2$; C, 59.99%; H, 5.49%; N, 12.72%.

Le spectre I.R. de cette substance est différent de celui du γ-(*p*-hydroxy)anilide de l'acide glutamique. La bande C = O est située à 5.93 μ, valeur caractéristique de la vibration C = O d'une γ-lactame tandis que la bande C = O de la fonction amide secondaire se trouve à 6.07 μ dans le spectre de la substance naturelle.

On retrouve cependant des bandes d'absorption situées à la même longueur d'onde, notamment à 6.25 et 6.64 μ, les deux bandes du noyau aromatique, à 3.05 μ la bande NH de l'amide secondaire et à 6.47 μ la bande II de l'amide secondaire.

Les spectres I.R. du phénol, du produit naturel, du (*p*-hydroxy)anilide de l'acide pyrrolid-2-one-5-carboxylique, de la tyrosine, du *p*-aminophénol présentent des bandes fort semblables aux environs de 7.3 μ et de 8.1 μ que l'on ne retrouve pas dans le spectre I.R. de la phénylalanine et qui semblent caractéristiques de la fonction phénolique. Les spectres ont été pris en phase solide.

La présente synthèse a donc donné le (*p*-hydroxy)anilide de l'acide pyrrolid-2-one-5-carboxylique et la réaction s'interprète de la façon suivante:



$$\mathrm{COOH{-}\underset{\displaystyle N}{CH}{-}CH_2{-}CH_2{-}C{=}O} + (p)\mathrm{HO{-}C_6H_4{-}NH_2} \dashrightarrow \mathrm{H_2O} + (p)\mathrm{HO{-}C_6H_4{-}NH{-}CO{-}\underset{\displaystyle NH}{CH}{-}CH_2{-}CH_2{-}C{=}O}$$

REMERCIEMENTS

Nous remercions très sincèrement le Centre de Recherche sur les Champignons et le Centre Interuniversitaire de Chimie Organique pour l'aide qu'ils nous ont apportée au cours de cette recherche.

RÉSUMÉ

Le γ (*p*-hydroxy)anilide de l'acide glutamique a été isolé à partir du champignon *Agaricus hortensis* par séparation chromatographique sur charbon activé et colonne de Dowex 1 × 8. Le nouveau composé donne par hydrolyse avec HCl 6 *N* de l'acide glutamique et du *p*-aminophénol, la nature de ces deux derniers étant confirmée par comparaison avec les substances synthétiques. La substance synthétisée à partir du *p*-aminophénol et de l'acide pyrrolid-2-one-5-carboxylique présente des propriétés identiques à celles de l'anilide naturel.

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STUDIES ON THE METASTABLE STATES IN THE RHODOPSIN CYCLE

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SUMMARY

Photic reaction of rhodopsin in the range of —195° to 20° has been examined by means of measurement of optical density at room temperature after illumination or re-illumination at low temperature. The experimental results are as follows:

1. The amount of rhodopsin remaining after illumination is constant in two regions of temperature (—195° to —140° and —80° to —20°).
2. When illuminated previously at —40°, the amount of rhodopsin is increased by re-illumination below —100°, presenting almost a constant value below —140°.
3. When rhodopsin solution illuminated at any temperature below —140° is re-illuminated at —195°, the amount of rhodopsin is identical with that of the non-re-illuminated; in the range above —100°, the former becomes more abundant than the latter.

On the basis of the experimental results described above, we have discussed the photochemical properties of the labile fraction of lumi- or meta-rhodopsin and the essential of the "liquid air illuminated rhodopsin".

INTRODUCTION

In previous work[1] it was found that a rhodopsin solution illuminated sufficiently in dry ice–acetone or liquid air still retained respectively 50–60 % or 60–80 % rhodopsin, as shown by measurement of 500 mμ absorption (difference) at room temperature. Furthermore, when a solution illuminated in dry ice–acetone was re-illuminated in liquid air, the amount of rhodopsin became more abundant than when illuminated in dry ice–acetone alone. (This phenomenon we called "photo-recovery"[2].) These facts appear to suggest that some metastable state such as being stable in liquid air is involved in the rhodopsin cycle. In order to confirm the existence of this metastable state, the photic reaction of rhodopsin in the range of —195° to 20° was investigated in detail.

METHODS

Two unsilvered Dewars of different sizes were combined for controlling the temperature of the rhodopsin solution under illumination (Fig. 1). According to the temperature, the outer Dewar was filled either with liquid nitrogen (—195° to —78°) or with water (—70° to 20°). A liquid filling the inside Dewar was chosen from the following series: Liquid nitrogen for —195°, liquid air for —186°, EPA-mixture (ether, isopentane and alcohol in volume ratio 8:3:5) for —170° to —80°, alcohol for —78° to 0° and water for 20°. Temperature of the inner liquid—regarded as that of the sample—was monitored with a thermometer (platinum resistance thermometer for —195° to —70°, toluene thermometer for —80° to 20°) within the error of ± 1°.

All the experiments were carried out under dim red light. Cattle rhodopsin solution was prepared by the same method as described in the previous paper[1]. Every solution was adjusted to pH 9.2 by addition of 1/20 *M* borax. Optical purity ($E_{400\,m\mu}/E_{500\,m\mu}$) : 0.3–0.5, optical density at 500 mμ : 0.25–0.4.

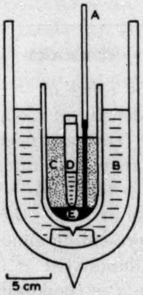

Fig. 1. Diagram of an apparatus for controlling temperature during illumination. A, thermometer; B, liquid nitrogen or water; C, liquid nitrogen, liquid air, EPA-mixture, alcohol or water; D, rhodopsin solution; E, mercury.

Before every experiment, a centrifuge tube containing 3 ml rhodopsin solution was immersed in liquid nitrogen and then placed in the inside Dewar with constant temperature. After being kept herein for more than 10 min*, the sample was laterally illuminated for 5 min by a 250 watt incandescent lamp (8,000 lux)**. Immediately after illumination, the sample was transferred into liquid nitrogen*** and, when

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* In a preliminary experiment, it was established that when the sample, which had been previously kept in liquid nitrogen, was immersed in alcohol of —20° it took about 5 min to reach a temperature equilibrium.

** In order to determine the suitable condition of illumination, we compared 8,000 lux × 5 min illumination with 8,000 lux × 10 min illumination with reference to the relative amount of residual rhodopsin measured at room temperature. As shown in the table, 8,000 lux × 5 min illumination and 8,000 lux × 10 min illumination resulted in almost equal values for every temperature during illumination. Therefore, we adopted 8,000 lux × 5 min as the condition of sufficient illumination in the present experiment.

TABLE I

Exposure time	*Temperature during illumination*		
	— 195°	*— 150°*	*— 100°*
5 min	82.7 %	80.5 %	61.8 %
10 min	82.1 %	80.3 %	59.6 %

*** A rhodopsin solution illuminated in dry ice–acetone showed no further change in the absorption spectrum, even though it was afterwards cooled to temperature of liquid nitrogen in the dark[2]. This fact indicates that liquid nitrogen is a suitable medium for the conservation of the sample.

necessary, thawed in a water bath. Thereafter, it was incubated at 23° in darkness for 1 hour. Optical density at 500 mμ was determined at room temperature (23°–28°) by a Beckman type spectrophotometer and the measurement was repeated again after rhodopsin had been completely bleached by illumination for 20 min with an incandescent lamp (8,000 lux). We regarded the difference of optical densities thus obtained as a measure of the amount of rhodopsin contained in the illuminated sample. Referring to the amount of rhodopsin likewise obtained from the dark sample kept in liquid nitrogen, we could calculate the relative amount of rhodopsin in %.

RESULTS

Experiment 1

In order to determine the relation between the amount of rhodopsin and the temperature under illumination, we carried out the following experiment. Rhodopsin solution was divided into a number (four to nine) tubes. One of them was kept at —195° in the dark, while any of the others was illuminated at a certain temperature between —195° and 20°.

The results are summarized in Fig. 2, where the relative amounts of rhodopsin after illumination at various low temperatures were plotted against the temperatures concerned. Fig. 2 indicates a general trend, namely that the amount of rhodopsin decreases as temperature goes up. This decrease, however, proceeds by no means uniformly, but stepwise. It should be noticed that the amount remains almost constant in two different temperature regions (—195° to —140° and —80° to —20°), which can probably be regarded as denoting two different states of rhodopsin illuminated at low temperature.

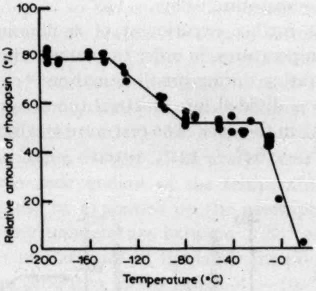

Fig. 2. Relationship between amount of rhodopsin and temperature of illumination.

Experiment 2

Assuming that Fig. 2 is suggestive of two different states in the course of the bleaching process of rhodopsin, one would be inclined to consider the "photo-recovery" as nothing but the transition from one state (caused by illumination of rhodopsin at —80° to —20°) to another (resulting from illumination of rhodopsin at —195° to —140°). Accordingly, one would expect the "photo-recovery" to be clearly revealed by re-illumination in the lower temperature range (—195° to —140°) after illumination in the higher range (—80° to —20°).

(*a*) As the temperature for pre-illumination —40° was adopted and as the temperatures for re-illumination various temperatures below —40°.

We divided the rhodopsin solution into twelve tubes containing 3 ml each. One of them was preserved at —195° in the dark as the basic sample. Five tubes were illuminated at —40° and preserved in liquid nitrogen. They were then re-illuminated at —195°, —161°, —131°, —100° and —70°, respectively. The corresponding five different temperatures and —40° were adopted for single illumination of the remaining six tubes.

The experimental results are shown in Fig. 3. We find that any sample re-illuminated at a temperature below —100° is more abundant in the relative amount of rhodopsin than that illuminated once at —40° (photo-recovery). Furthermore, the amount of rhodopsin in the sample re-illuminated at any temperature lower than —131° is considered as almost constant (68 %), while the sample re-illuminated above —131° denotes a decrease in the amount of rhodopsin in accordance with the rise in temperature for re-illumination, until the re-illumination at —70° seems to become almost ineffective.

This result indicates to us that a considerable increase in the amount of rhodopsin (photo-recovery) is caused by re-illumination in the temperature range between —195° and —140°, where a metastable state suggested by Fig. 2 would be formed on illumination.

On the other hand, every sample re-illuminated at certain temperature from —195° to —100° (solid circle) indicates evidently a lower value in the relative amount of rhodopsin than that illuminated once at the corresponding temperature (open circle). This fact demonstrates that the state of rhodopsin illuminated at —40° cannot always be completely changed by re-illumination to the state formed by single illumination at temperature below —140°.

(*b*) We carried out a further experiment of re-illumination at —195° after illumination at various temperatures, in order to examine the relation of the amount of rhodopsin to the temperature during pre-illumination.

Rhodopsin solution was divided into thirteen tubes containing 3 ml each. One of them was kept at —195° in the dark. The rest were treated as six pairs. Each pair was illuminated at —186°, —162°, —131°, —100°, —70° and —39°, respectively,

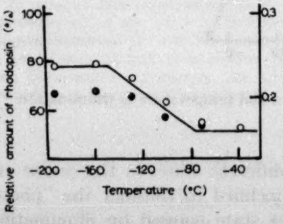

Fig. 3. Relationship between amount of rhodopsin and temperature of re-illumination. Solid circles: re-illuminated at various temperatures after illumination at —40°. Open circles: illuminated once at the corresponding temperatures. Solid line: from Fig. 2.

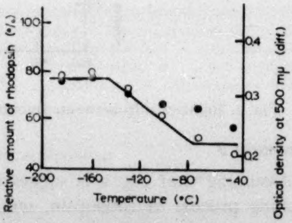

Fig. 4. Relationship between amount of rhodopsin and temperature of pre-illumination. Solid circles: re-illuminated at —195° after illumination at various temperatures. Open circles: illuminated once at the corresponding temperatures. Solid line: from Fig. 2.

and then preserved in liquid nitrogen. One of each pair was subsequently re-illuminated at—195°.

As Fig. 4 shows, below —131° scarcely any difference is found between amount of rhodopsin in the re-illuminated sample (solid circle) and in the control (open circle); above —100°, however, the former becomes obviously more abundant than the latter.

An increase in the amount of rhodopsin (photo-recovery) was most clearly revealed by the samples pre-illuminated at —39° and —70°. This fact suggests to us that illumination in the temperature region between —80° and —20° would produce one state of rhodopsin with reference to the "photo-recovery". Illumination in the temperature region between —195° and —140° is considered as producing another state of rhodopsin, on the basis of the fact that the re-illumination at —195° is ineffective.

DISCUSSION

From the spectroscopic study of rhodopsin at low temperature, WALD, DURELL AND ST. GEORGE[3] found that two different states of rhodopsin, named lumi- and meta-rhodopsin, were demonstrable in the decomposing process at low temperature. Lumi- and meta-rhodopsin are considered to correspond to our rhodopsin illuminated at —70° and at —20°, respectively. However, we obtained the experimental result that when a rhodopsin solution is illuminated at any temperature in the range of —80° and —20° almost a constant value for the relative amount of rhodopsin is obtained. (Fig. 2). We also established in the previous work[4] that lumi-rhodopsin could not be distinguished from meta-rhodopsin with reference to the amount of photo-recovered rhodopsin. Therefore, lumi-rhodopsin can be considered to be identical with meta-rhodopsin so far as our experiments are concerned, and both of them may be involved in a single state of rhodopsin illuminated at any temperature from —80° to —20°.

HUBBARD AND KROPF[5] considered that lumi- or meta-rhodopsin is a steady-state mixture, which is composed of rhodopsin, iso-rhodopsin and such labile fractions as are stable below —15°, and that this labile fraction is regenerated to rhodopsin and iso-rhodopsin by the action of light. If we accept their opinion, the fact that the amount of rhodopsin was independent of the temperature of illumination in the range —80° to —20° must be explained on the assumption that every rhodopsin solution illuminated at any temperature between —80° and —20° (lumi- or meta-rhodopsin) contains an equal amount of the labile fraction in this range of temperature. The labile fraction produced in the range of —100° and —40° must be converted into rhodopsin (or iso-rhodopsin) by re-illumination at —195°, since we could observe the increase in the relative amount of rhodopsin (Fig. 4). This fact probably supports the view of HUBBARD AND KROPF that the labile fraction is regenerated to rhodopsin and iso-rhodopsin by the action of light.

We, on the other hand, found the following: (1) the relative amount of rhodopsin was independent of the temperature of illumination in the range —195° to—140° (Fig. 2); (2) when the solution previously illuminated at —40° was re-illuminated below —140°, the amount of rhodopsin increased towards a constant value (68%) (Fig. 3); (3) with a solution illuminated below —140° there was no photo-recovery on re-illumination at —195° (Fig. 4). In view of these results, the temperature —140°

attracts our attention with regard to thermal stabilization of the metastable state formed in the photo-decomposing process of rhodopsin. The "liquid air illuminated rhodopsin" which was proposed in a previous paper[2] may be considered as a short-lived state, being stable below —140°, and lying between rhodopsin and lumi-rhodopsin in the rhodopsin cycle. "Liquid air illuminated rhodopsin" is considered to be partly changed to the labile fraction (component of lumi- or meta-rhodopsin) in the dark by warming it to approx. —40°. On the other hand, lumi- or meta-rhodopsin may not be converted to "liquid air illuminated rhodopsin" when cooled to —195° in the dark[2]; the change would occur only on exposure at —195°.

We cannot yet decide at present whether "liquid air illuminated rhodopsin" is a single intermediate or a steady-state mixture. On the basis of the view that lumi- or meta-rhodopsin is a steady-state mixture, "liquid air illuminated rhodopsin" may also be regarded as another steady-state mixture, differing from lumi- or meta-rhodopsin in the property of the labile fraction. In the field of radiochemistry, some free radicals are found to be stable below —140°[6,7]. This suggests that "liquid air illuminated rhodopsin" may be a kind of free radical. Our future research will be conducted along these lines.

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Short Communications

Hydrolysis of thiamine phosphates by phosphatases in rat liver

In the normal rat liver the supernatant fraction contains nearly twice as much TDP as the mitochondrial fraction[1-3], and more than 15 times as much thiamine[3]. The nuclear fraction is very poor in TDP[1-3].

Repeated (4–6 times) injection of the rats with CCl_4 decreases the content of the supernatant TDP (see ref. 2, 3). This is also the case in the liver supernatant from rats fed ethanol for several months[4]. An increased dephosphorylation of TDP, induced by the treatments, was suggested to be the cause of the above decrease[3-4].

The enzymic dephosphorylation of TDP has been further investigated, and in this brief note information is given about the enzymic activity in different liver cell fractions, pH optima, and the effect of pretreating the animals with CCl_4.

Liver cell fractions were prepared according to SCHNEIDER AND HOGEBOOM[5]. The nuclear fraction was then resuspended, homogenized, and centrifuged twice more before incubation. The incubation medium contained 15 μmoles Mg^{2+}, 0.5 μmole [^{32}P]TDP, equally labelled in both phosphate groups, or 0.5 μmole [^{32}P]TMP, tissue extract or crude enzyme corresponding to 80 mg liver, and 0.1 *M* Tris–maleate or glycine–NaOH buffer to a final volume of 3 ml. The hydrolysis of the thiamine phosphates was calculated as per cent of the total radioactive phosphate occurring as inorganic phosphate after the incubation.

Incubation of TDP with cell fractions from rat liver. Radioactive TDP was incubated for varying times with supernatant (containing microsomes), mitochondria, or nuclei from normal and CCl_4-treated rats. All fractions were found to contain an enzyme decomposing TDP, the optimum pH being 9.6. In mitochondria also a second optimum at pH 8.6 was found. The supernatant fraction was about ten times as active as the mitochondrial and the nuclear fractions. CCl_4 treatment of the animals strongly increased the enzymic dephosphorylation of TDP in the supernatant (Fig. 1B) and mitochondrial fractions but only slightly in the nuclear fraction.

Paper chromatographic analyses showed that TMP was the main product of the TDP decomposition. Only traces of thiamine could be detected on the chromatograms, irrespective of whether the incubation had been performed at a pH around 7 or as high as 9.6.

Incubation of TMP with cell fractions from rat livers. When radioactive TMP was incubated with the supernatant fraction in the same way as described for TDP the optimum pH for its hydrolysis was found to be 6.1. No significant increase was caused by previous CCl_4 treatment of the rats (Fig. 1A).

Incubation of TDP and TMP with crude enzyme preparations from rat liver. In order to make sure that the pH effect on the rates of decomposition of TDP and

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Abbreviations: TDP, thiamine diphosphate; TMP, thiamine monophosphate; Tris, tris(hydroxymethyl)aminomethane.

TMP, respectively, was not caused by a pH dependent release of phosphatase(s) from subcellular particles, crude enzyme preparations were made from normal rat liver in either of two ways: as a $KHCO_3$ extraction of a liver acetone powder, described by GIBSON *et al.*[6] for isolation of nucleoside diphosphatase from mitochondria, and by acetate extraction and treatment with butanol as used by MORTON[7], when isolating intestinal alkaline phosphatase.

In either case we found the preparations to be very active against TDP, transforming it into TMP and with only a slightly altered pH optimum as compared with that of the cell fractions (Fig. 1D).

With the acetone powder extract an obvious hydrolysis of TMP was obtained with an optimum pH at 5.8 (Fig. 1C), whereas the activity against TMP was almost negligable with the second preparation.

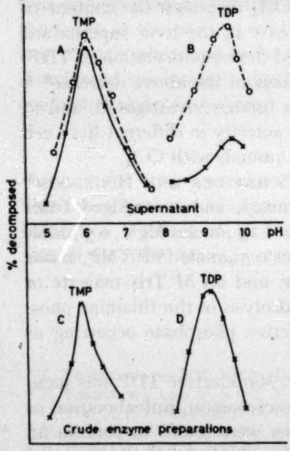

Fig. 1. Effect of pH on the activity of phosphatases hydrolysing TDP and TMP. A and B, effect of pH when TDP and TMP are incubated with the supernatant fraction from rat liver; C and D, influence of pH when incubated with crude enzyme preparations from rat liver. Continuous lines, enzymes from normal rats; lines of short dashes, enzymes from CCl_4-treated rats. Additions: see text; time, 30 min; temp. 37°. The reaction was stopped by addition of H_2SO_4 to a final concn. of 0.5 *M*, and inorganic phosphate separated from organic according to ERNSTER *et al.*[8].

The facts that both crude enzyme preparations hydrolysed TDP into TMP, whereas only one transformed TMP into thiamine, and that TDP and TMP are hydrolyzed at a maximum rate at widely different pH's strongly indicate that the two steps are catalyzed by different phosphatases.

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Enzymic studies on dystrophic mice and their littermates (lipogenesis and cholesterolgenesis)

This laboratory has been carrying out investigations using mice with muscular dystrophy and their littermates in attempts to define biochemical lesions[1–3]. Systematic enzyme assays have been performed utilizing tissues of both mice. The hereditary disease of these mice in many respects resembles human muscular dystrophy[4]. The enzyme systems to be reported are the ones concerned with lipogenesis and cholesterolgenesis[5,6].

Male dystrophic mice and their corresponding littermates were purchased during the winter from the Jackson Memorial Laboratory, Bar Harbon, Maine. The animals used were 5 to 6 weeks old. In all cases the dystrophic mice exhibited malfunction of the hind legs. The normal littermates were adjudged to be in satisfactory health by physical observation. The animals received an *ad libitum* diet of Purina chow and water. Attempts to utilize frozen sections for the study of these enzymes gave erratic results. For the above reason all the animals used were sacrificed prior to the enzymic assay. The brain, liver, kidney, spleen, and museles from each individual animal were weighed and then homogenized in a loose fitting Potter-Elvehjem glass homogenizer. The crude homogenates were freed of debris by centrifugation for 6 min at 500 × *g*, and were incubated for 3 h with [^{14}C-*Me*]acetate obtained from a commercial source[*] (1 mC/mmole). The homogenates were previously fortified with 1 mg each of AMP, TPP, and DPN and were incubated under 100 % O_2. After incubation, carrier palmitic acid and cholesterol were added to each preparation. The cholesterol and fatty acids were recovered[5,6]. Digitonides and calcium salts were then used to purify the materials. The cholesterol-digitonide was then converted to the dibromo derivative and the calcium salts of the fatty acid recovered as free fatty acids. The recovered materials were radiometrically assayed in a flow counter to a standard deviation of ± 0.3 % and corrected for self-absorption.

TABLE I

LIPOGENESIS AND CHOLESTEROLGENESIS IN "NORMAL" AND "DYSTROPHIC-MICE" TISSUE

	Recovered radioactivity (Average of 22 pairs of mice; Dystrophic and Littermate)			
	Cholesterolgenesis		*Lipogenesis*	
Organ	*Counts/min/mg C/mg N of original tissue from dystrophic mice*	*Percentage ratio of radioactivity of dystrophic to littermate*	*Counts/min/mg C/mg N of original tissue from dystrophic mice*	*Percentage ratio of radioactivity of dystrophic to littermate*
Brain	8650	231	6430	187
Liver	7840	194	8970	197
Kidney	3490	184	3420	205
Spleen	4850	171	8440	201
Skin	6730	159	9730	219
Muscle	86	118	385	131

The radioactivity found for the recovered cholesterol and fatty acid of "littermates" has been designated as 100 % while the recovered radioactivity in cholesterol

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Abbreviations: AMP, adenosine monophosphate; TPP, thiamin pyrophosphate; DPN, diphosphopyridine nucleotide.

[*] All radioactive materials were obtained on allocation from the United States Atomic Energy Commission.

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and fatty acid for the dystrophic animal was the percentage obtained after comparison with values of the "normal littermate". These results are in Table I and represent the results obtained from 22 pairs of dystrophic mice and their corresponding littermates. The percentage standard deviation of the mean observed throughout these experiments was ± 21 %. The observed ratios are thus statistically significant except for muscle.

From Table I it can be concluded that homogenized and fortified tissues of dystrophic mice have a greater lipogenesis and cholesterolgenesis than their "normal" littermates. The brain shows the greatest difference, and is followed closely by the liver and kidney. The spleen and skin showed smaller increments. All values presented have been corrected to equal concentrations of nitrogen present in the original homogenate.

These results suggest that definite metabolic differences occur in the various fat-metabolizing centers of the dystrophic mouse.

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Direct ion-exchange chromatography of tissue extracts without precipitation of protein by use of non-ionic detergents

In general, the separation of constituents of a biological preparation by ion-exchange chromatography is done with protein-free filtrates because protein adheres to ion-exchange resins and clogs the columns. In the course of other experimental work, one of us (J.E.S.) chanced to discover that proteins in the presence of certain polyoxyethylene non-ionic detergents did not adhere to ion-exchange resins but flowed through resin columns directly. Small ionized molecules present in such protein-detergent mixtures did not seem to be affected and were retained by the resin column from which they could be afterward displaced sequentially in the usual manner, thereby permitting direct chromatography of tissue extracts.

Abbreviations: OPE_x, *p-t,t*-octylphenoxy-polyoxyethylene-ethanol of *x* ethylene oxide units; MDTA, methyldodecylbenzyltrimethyl-ammonium chloride; UMP, uridine monophosphate; TPN, triphosphopyridine nucleotide; IMP, inosine monophosphate; AMP, adenosine monophosphate; GMP, guanosine monophosphate; UDP, uridine diphosphate; ADP, adenosine diphosphate; UTP, uridine triphosphate; ATP, adenosine triphosphate; UDPG, uridine diphosphoglucose; PNA, pentose nucleic acid.

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The detergents used were a series of *p-t,t*-octylphenoxypolyoxyethylene-ethanols containing 9 to 16 ethylene oxide units per molecule (obtained from Rohm and Haas, Inc., Philadelphia) and some thioether analogs (obtained from Monsanto Chemical Company, St. Louis). Polymer species of specific chain length were obtained by chromatographic separation on silica columns[1]. Detergent concentrations of 0.05 % or more were required; these concentrations are somewhat greater than the critical concentration of detergent at which micelles are formed[2]. For routine chromatography of tissue extracts, crude fractions containing a mixture of several adjacent polymer species were found to be adequate*.

Excised rat tissue, frozen in liquid nitrogen and powdered, was added to 9 vol. 3 % ice-cold detergent solution and mixed thoroughly in a Waring Blendor. Enzyme activity continued in tissue preparations containing OPE detergent alone leading to changes in the constituents. To minimize enzymic alteration, about 0.3 % of a cationic detergent, methyldodecyl-benzyl-trimethylammonium chloride (obtained from Rohm and Haas, Inc., Philadelphia), or 4–8 *M* urea was added. An insoluble residue containing about 20 % of the total protein remained. The amount of insoluble protein and structural elements could be lessened by the use of urea in the homogenizing medium in addition to detergent. At times, the chromatographic columns were clogged by cell particulates. This was avoided by filtration through a coarse fritted glass disc or by sonication of the homogenate and centrifugation at 14,000 × *g* for 16 min at 0° prior to chromatography. For comparison, $HClO_4$ extracts were prepared according to the directions of HURLBERT *et al.*[3].

A typical direct chromatogram of the free nucleotides of a rat-liver homogenate is shown in Fig. 1, together with a chromatogram of the acid-soluble fraction of a rat-liver homogenate obtained after $HClO_4$ precipitation of protein[3]. While the positions of the nucleotide peaks in the two chromatograms correspond, the total quantity of nucleotides obtained in the direct chromatogram was much less than was obtained with the $HClO_4$ extract. Probably this was due to liberation of nucleotides bound to protein by $HClO_4$ while the detergent did not rupture these protein complexes. This assumption was supported by the observation that additional free nucleotides were found upon chromatographing the $HClO_4$-treated effluent of a direct chromatogram. Though certain protein complexes were broken by the detergent, it often provided a means of separating protein complexes from free ligand. For example, detergent and ion-exchange resins have been used for separating protein-bound iodine from inorganic iodide in the determination of iodine bound to serum protein[4].

The resolution of peaks in direct chromatography is less sharp than in chromatography of protein-free filtrates. The loss of resolution seems to be associated with the presence of protein, since addition of detergent and urea to protein-free filtrates resulted in chromatograms identical in all respects to those obtained in the absence of detergent. Furthermore, fractions obtained from direct chromatograms (which contained neither detergent nor protein) showed the same resolution and separation of component peaks upon rechromatography as fractions from $HClO_4$ filtrates.

Direct chromatograms using detergent of high-speed supernatant fractions of rat liver showed only minor differences in the free nucleotide pattern when compared

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* Two useful crude fractions (and their average ethylene oxide chain length) obtained from Rohm and Haas, Inc., Philadelphia, were: Triton X-100 = OPE_{av-10} and Triton X-165 = OPE_{av-16}.

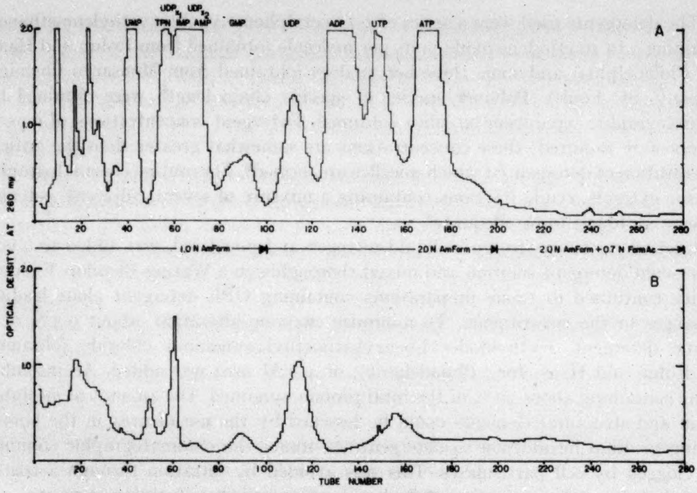

Fig. 1. (A) Chromatogram of $HClO_4$: filtrate of 15 g rat liver on Dowex 1-X10, 200–400 mesh resin gradient eluted with an ammonium formate system at pH 5.0. (B) A similar direct chromatogram of rat liver homogenized in 9 parts 3.0 % OPE_{11}, 0.3 % MDTA in 0.35 M sucrose.

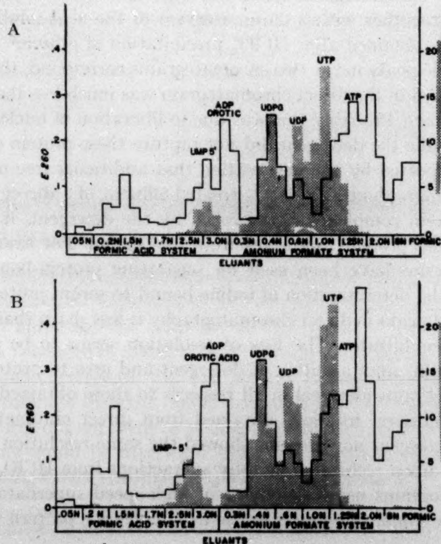

Fig. 2. (A) Chromatogram of protein-free filtrate of high-speed supernatant of rat liver incubated for 90 min with [^{14}C]orotic acid at 37°. (B) A similar direct chromatogram of high-speed supernatant using 0.5 % OPE_{11} final concentration after identical incubation with [^{14}C]orotic acid.

with chromatograms of $HClO_4$ filtrates (Fig. 2.) Direct chromatography was particularly useful for the separation of unstable phosphate esters such as phosphocreatine and phosphoglycocyamine; gradient elution with an ammonium formate system at pH 7.2 was used.

No evidence for an interaction between the detergent and ion-exchange resins was found. Titration curves of Dowex 1-X8, 100 to 200 mesh resin in the hydroxyl cycle suspended in water gave an ion-binding capacity of 3.06 mequiv./g wet wt. resin when titrated with HCl and 3.16 mequiv./g in 3.0 % OPE_{11}. In order to evaluate the behavior of this resin with ionic aggregates, it was titrated with *p*-dodecylbenzenesulfonic acid (obtained from the Continental Oil Co., Chicago), a strongly acidic detergent which forms micelles in water. Titration in water gave an apparent ion-binding capacity of 1.98 mequiv./g; in the presence of 3.0 % OPE_{11}, the apparent resin capacity was 1.94 mequiv./g. The differences observed were not considered significant.

A change in specific resin volume often is observed when an ion-exchange resin is put into a medium with which it reacts. No change in resin volume was observed when Dowex 1 or Dowex 50 previously suspended in water was transferred to a medium containing 3.0 % OPE_{11}. A study of the protein interaction is in progress and the results will be the subject of a future communication.

Direct chromatography using OPE detergents appears to be a technique suited for the separation of labile constituents of tissue preparations requiring mild treatment to prevent decomposition. The method is applicable to all substances of low molecular weight that can be separated by ion-exchange chromatography. Protein-bound complexes are likely to remain intact and the ligand not removed by the ion-exchange resin in the presence of detergent.

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Preliminary Notes

Metabolism of the white blood cells in maple-syrup-urine disease

Maple-syrup-urine disease is an inherited metabolic anomaly characterized clinically by early onset of feeding problems and neurological symptoms with eventual mental retardation and death, and a maple-syrup odor to the urine. It has been suggested that the biochemical defect may be at the step involving oxidative decarboxylation of the branched-chain keto acids of the respective amino acids: leucine, isoleucine and valine. This was based on finding an elevation in the branched-chain amino acids in the plasma, normal transaminase activity in tissues obtained at post-mortem, the accumulation of branched-chain keto acids in the urine and the absence of an excess of metabolites located further down the degradative pathway[1-3]. The enzymes necessary for the oxidative decarboxylations of the branched-chain keto acids are widely distributed in animal tissues including the white blood cell[3]. This suggested that the enzymic defect might be directly demonstrable in the white blood cell of the peripheral blood of a patient.

The opportunity to test this hypothesis presented itself with the birth of a sibling to the previously reported case. The patient revealed the typical biochemical abnormalities by the second week of life. At the time of these studies she was 1 year old and had been on a synthetic amino acid diet since the diagnosis had been made. The plasma levels of the branched-chain amino acids were within normal limits; methionine, however, was elevated. There was no increased excretion of keto acids[4].

Blood was drawn into a heparinized syringe and the red blood cells sedimented with fibrinogen. The supernatant was centrifuged and the white blood cells resuspended in sodium phosphate buffer. Aliquots were transferred to 10-ml Erlenmeyer flasks with a center well. The number of white blood cells per flask approximated that which had been present in 1 ml of plasma. The incubation medium consisted of 0.8 ml 0.067 M sodium phosphate buffer, pH 7.4, 0.03 ml 0.15 M $MgSO_4$, and 2 μg of crystalline catalase (100 units/mg). To this was added one of three substrates: 150,000 counts/min DL-[1-^{14}C]leucine (30 μg), 250,000 counts/min L-[^{14}C]isoleucine (322 μg) or 150,000 counts/min DL-[1-^{14}C]valine (14 μg). The center well contained 0.1 ml 1 % Na_2CO_3 in 1 N KOH. Control flasks contained all the above except for the white blood cells. The flasks were incubated with shaking for 75 min at 37°.

Following incubation the contents of the center well were precipitated as $BaCO_3$, washed and transferred to weighed planchettes for detection of radioactivity. The total amount of $^{14}CO_2$ was calculated by relating the weight of $BaCO_3$ on the planchette to the amount of carrier carbonate added to the center well. Results were corrected for self-absorption.

The incubation medium was treated with 2,4-dinitrophenylhydrazine, extracted into *m*-xylene, re-extracted into Na_2CO_3 which was acidified and finally extracted into ethyl acetate for transfer to a planchette. Radioactivity was determined in a gas-flow counter. This is reported as the "keto acid" fraction.

The results are presented in Table I. The "keto acid" fraction is probably represented primarily by branched-chain keto acids. This was verified for leucine by paper chromatography in the patient and in one control case. The presence of small amounts of other organic acids cannot be excluded. The formation of "keto acids" is about the same in the normal infant and in the patient indicating active transamination. However, the accumulation of $^{14}CO_2$ by the patient's cells was drastically reduced for all three amino acids indicating grossly deficient or absent decarboxylation. This supports the hypothesis that the three amino acids share one enzyme in the degradative pathway which is deficient in maple-syrup-urine disease. It excludes the possibility that only one amino acid was primarily involved and that the accumulation of this keto acid interfered secondarily with the decarboxylation of the other two.

TABLE I

TRANSAMINATION ("KETO ACIDS") AND DECARBOXYLATION (CO_2) IN MμMOLES AS DETERMINED IN THE WHITE BLOOD CELLS OF NORMAL 1-YEAR-OLD INFANTS AND IN A PATIENT WITH MAPLE-SYRUP-URINE DISEASE

		Leucine		*Isoleucine*		*Valine*	
		"Keto acids"	*CO_2*	*"Keto acids"*	*CO_2*	*"Keto acids"*	*CO_2*
Control infants	1	1.86	1.80	2.0	7.4	0.31	0.77
	2	2.47	1.47	3.0	3.7	0.03	0.42
	3	1.72	0.62	2.9	1.7	0.12	0.83
	4					0.20	0.30
Maple-syrup-urine disease	5	1.50	0.01	3.0	0	0.46	0.03

The diagnosis in the present case of maple-syrup-urine disease was delayed for almost two weeks after birth because the urine failed to show an appreciable accumulation of keto acids. It was therefore desirable to learn whether this enzymic function could be demonstrated in the white blood cell of the young infant. Four infants were tested with [1-^{14}C]leucine (3 at 2½ days and 1 at 6 days of life). The production of $^{14}CO_2$ was 0.87, 1.71, 0.43 and 0.38 mμmoles, respectively. It therefore seems possible to make an early and specific diagnosis of maple-syrup-urine disease by studying the metabolism of the peripheral white blood cell.

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Glucuronide formation in the active transport of amino acid and thyroxine analogues by rat intestine

It has recently been demonstrated that L-[^{131}I]MIT is actively transported *in vitro* from the mucosal to the serosal side of everted sacs of rat small intestine[1]. Subsequent experiments with ^{14}C-labeled amino acids have suggested that MIT is transported by a mechanism which is also responsible for the transport of the naturally occurring amino acids.

In the course of this investigation it has been found that the propionic and acetic acid analogues of thyroxine and triiodothyronine are likewise transferred from the mucosal to the serosal side of the small intestine against a concentration gradient. The active transport of one of these compounds, [^{131}I]TRIAC, has been studied extensively. All the TRIAC which accumulates on the serosal side of the intestinal wall is in the form of a conjugate. This conjugate is acid-labile, alkali-stable, and does not form a hydroxamate derivative when incubated with NH_2OH[2]. The conjugate is cleaved by incubation for 2 h at 37° with β-glucuronidase (250 units/ml); this breakdown can be prevented by the addition to the incubation medium of 1,4-saccharolactone, a specific inhibitor of β-glucuronidase[3]. The conjugate is not altered by incubation for 18 h with Taka-diastase, an enzyme which cleaves a sulphate conjugate[4]. A positive reaction is given by the naphthoresorcinol method for determining glucuronic acid[5]. This evidence suggests that the conjugate is an ethereal glucuronide linked through the phenolic hydroxyl of the TRIAC. This glucuronide of TRIAC is not itself actively transported by the rat intestine. Indeed, the mucosal membrane is apparently as impermeable to TRIAC glucuronide as it is to salicyl glucuronide[6]. This relative impermeability to the conjugate offers a reasonable basis for its accumulation on the serosal surface of the everted gut sac.

These findings with thyroxine analogues prompted a re-examination of the inner fluid from gut sacs incubated with MIT. It had been reported[1] that a portion of the radioactivity in the inner fluid from sacs incubated with MIT migrates in *n*-butanol–acetic acid with an R_F of less than 0.1 (*i.e.*, with an R_F less than that of either iodide or MIT). Subsequent experiments have demonstrated that this radioactive material is in a conjugate of MIT and that from 5–35 % of the MIT inside the sac may be recovered in this form. As with TRIAC, the conjugate of MIT is acid-labile and alkali-stable; it does not form a hydroxamate derivative with NH_2OH; it is broken down by β-glucuronidase and gives a positive reaction with naphthoresorcinol. It is concluded that the conjugate is a glucuronide, probably linked through the phenolic hydroxyl of MIT. As with the TRIAC glucuronide, the MIT glucuronide is not actively transported; indeed, the gut wall is also relatively impermeable to this conjugate.

The mechanisms responsible for the active transport of MIT and of TRIAC by everted gut sacs are apparently similar, if not identical. Both systems have the same pH optima, are stimulated by the same substrates, and inhibited by the same inhibitors. It is of particular interest that the transport of TRIAC is inhibited by amino acids; as can be seen in Fig. 1 for L-phenylalanine, this inhibition is competitive in nature. Studies of MIT transport have shown that the α-amino group is the one part

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Abbreviations: MIT, monoiodotyrosine; TRIAC, triiodothyroacetic acid.

of the molecule which is absolutely essential for its transport[1]. These findings suggest that the active transport of MIT may be accomplished through the production of two glucuronides. Evidence for the formation of an ethereal glucuronide has been presented. It has not been possible to obtain direct evidence for the production of an N-glucuronide of MIT; however, all of the evidence suggests that such a conjugate is formed. Those N-glucuronides which have been previously described have been so labile that they could not be recovered by chromatographic techniques used in this study[7]. Such glucuronide formation would offer an attractive explanation for the active transport of MIT and other amino acids by rat intestine.

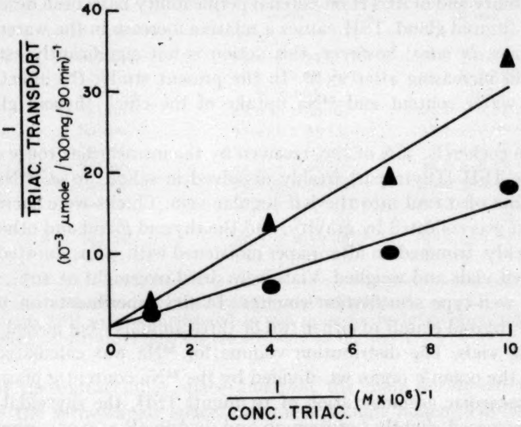

Fig. 1. Lineweaver–Burk plots of TRIAC transport in the presence and absence of inhibitor. ●, no inhibitor; ■, 0.04 *M* L-phenylalanine.

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Enhancement of thyroidal water content and uptake of ^{24}Na after TSH administration

The concept has emerged in recent years that hormones may function by altering the permeability of a target tissue to various substances[1]. Early effects of estrogen on uterine permeability and of ACTH on adrenal permeability have been described[2-4]. In the case of the thyroid gland, TSH causes a relative increase in the water content of beef thyroid slices *in vitro*; however, this action is not significantly established for 10 h and is still increasing after 25 h[5]. In the present study, the effect of TSH *in vivo* upon the water content and ^{24}Na uptake of the chick thyroid gland was explored.

White leghorn cockerels, 36 h of age, received by the intracardiac route 0.1 ml of saline or of bovine TSH (Thytropar) freshly dissolved in saline. 10 μC ^{24}NaCl were injected in a volume of 0.1 ml into the left jugular vein. Chicks were sacrificed by decapitation, blood was collected by gravity, and the thyroid gland and other tissues were dissected quickly, trimmed on filter paper moistened with saline, blotted lightly, placed in tared shell vials and weighed. Vials were dried overnight at 105°, weighed and counted in a well-type scintillation counter. In the experiments on thyroidal water content, the thyroid glands of either two or three animals were pooled in glass-stoppered weighing vials. The distribution volume for ^{24}Na was calculated as the content of ^{24}Na in the organ/g organ wt. divided by the ^{24}Na content/g plasma.

After the intracardiac administration of 10 munits TSH, the thyroidal concentration of water increased slightly between 20 and 40 min ($P < 0.05$), significantly by 60 min ($P < 0.01$) and maximally between 180 and 240 min. The effect was still evident, although submaximally, 18 h later. The water concentration of saline-treated control chicks averaged 72.3 mg/100 mg thyroid weight; the maximal response to TSH resulted in an increase of approximately 4.0 mg/100 mg.

^{24}Na penetrated the thyroid gland slowly in the absence of added TSH; its volume of distribution was only 29.1 % of thyroid weight in 20 min, but had risen to 51.0 % by 180 min. By contrast, ^{24}Na reached its maximal volume of distribution within 20 min in all other tissues studied (Table I). The administration of TSH accelerated the entry of ^{24}Na into the thyroid gland while the ultimate volume of distribution was not significantly increased. TSH did not alter the ^{24}Na uptake of any of the nine extrathyroidal tissues studied.

Employing a fixed 20-min interval between ^{24}Na and sacrifice, the time-response pattern of the TSH effect was studied. When TSH was administered only 1 min before ^{24}Na, no enhancement of ^{24}Na uptake was induced. When the interval was 10 min, the volume of distribution rose suddenly from a control value of 27.9 % of thyroidal weight to 45.0 %. The maximal response continued when a 20-min interval was allowed, but by 60 and 100 min, the differences between the TSH-treated and saline-treated groups were barely significant, and at 220 min the effect had disappeared.

These preliminary observations suggest that an obstruction to the free passage of sodium exists at one or more thyroidal membranes in the untreated young chick. Since a volume of distribution of nearly 30 % of thyroid weight is achieved rapidly, it appears likely that this component represents for the most part the intravascular

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Abbreviations, TSH, thyroid-stimulating hormone (thyrotropin); ACTH, adrenocorticotropic hormone.

TABLE I

THE EFFECT OF TSH ON THE RAPIDITY OF DISTRIBUTION OF ^{24}Na INTO THE THYROID GLAND AND OTHER TISSUES OF THE CHICK

Tissue	*Substance injected*	*Volume of distribution at various times after ^{24}Na (g/100 g fresh tissue)*		*P value (20 min vs. 180 min)*
		20 min	*180 min*	
Thyroid	Saline	29.1 ± 1.37 (11)*	51.0 ± 1.35 (12)	< 0.001
	TSH**	43.6 ± 2.30 (12)	54.7 ± 2.49 (12)	< 0.01
	P value	< 0.001	> 0.05	
Adrenal	Saline	28.0 ± 0.99 (6)	29.8 ± 0.44 (6)	> 0.05
Duodenum	Saline	33.4 ± 1.35 (5)	35.8 ± 0.60 (6)	> 0.05
Fat	Saline	19.7 ± 2.65 (5)	17.5 ± 2.23 (6)	> 0.05
Kidney	Saline	42.7 ± 0.90 (6)	42.8 ± 0.76 (6)	> 0.05
Liver	Saline	23.8 ± 0.58 (6)	23.6 ± 0.49 (6)	> 0.05
Muscle	Saline	67.1 ± 2.06	66.7 ± 2.32 (6)	> 0.05
Stomach	Saline	29.5 ± 0.49 (6)	30.4 ± 1.18 (6)	> 0.05
Testis	Saline	32.5 ± 0.50 (6)	31.9 ± 0.76 (5)	> 0.05
Thymus	Saline	18.7 ± 0.61 (5)	17.8 ± 0.79 (6)	> 0.05

* Mean ± standard error of the mean (number of animals).

** 10 munits TSH were administered 20 min prior to the intravenous injection of ^{24}Na. Data on TSH effect on extrathyroidal organs are omitted for brevity.

fluid and the extracellular space and that the more slowly-entered compartments comprise the intracellular space and the follicular lumen. The effect of TSH is to increase the rate of entry of ^{24}Na into the thyroid gland. It is not possible from the available data to determine which of the above-mentioned four compartments or which of three intercompartmental membranes (capillary wall, basal cell membrane, apical cell membrane) is altered by the administration of TSH. That the increase in ^{24}Na content induced by TSH is not due to an expansion of intrathyroidal vascular volume is suggested by a preliminary experiment in which the thyroidal content of radio-iodinated serum albumin was not enhanced by TSH. The onset of the effect of TSH on ^{24}Na uptake coincides temporally with its effect on the release of pre-labeled thyroidal iodine-containing compounds[6]; it is possible, therefore, that Na^+ is somehow involved either in the enzymic proteolysis of thyroglobulin or in the transport of the resultant products from the lumen of the follicle to that of the capillary. Since TSH induced a maximal rise in water content only after the rise in sodium uptake had disappeared, the former would appear to be a secondary phenomenon.

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Biodegradation of dehydro-L-ascorbic acid; 2,3-diketo-aldonic acid decarboxylase from rat liver

Decarboxylation of L-ascorbic acid at C-1 *in vivo*[1,2] and *in vitro*[1,3-5] has been shown to take place after its oxidation to DAA followed by its hydrolysis to DKG. The product of decarboxylation is reported as L-xylose[1] in liver or L-lyxonic acid[4] in kidney. The present communication deals with an enzyme, 2,3-diketo-aldonic acid decarboxylase, which was purified to confirm the reaction mechanism and the products of above decarboxylation. The decarboxylation activity was determined manometrically in 0.1 M phosphate buffer, pH 6.8, with 20 mM of DKG added from the side arm, at 37° under N_2. The enzyme activity was also estimated by the disappearance of DKG (0.2 mM in 0.01 M phosphate buffer) with 2,4-dinitrophenylhydrazine or with aniline citrate.

The supernatant fraction of rat-liver KCl homogenate was subjected to $(NH_4)_2SO_4$ fractionation (0.55–0.70 satn.), dialysis, and fractionation on a DEAE-cellulose column. The fraction eluted with 0.05 M NaCl in 5 mM phosphate buffer, pH 7.5, usually showed a specific activity 100 times that of the supernatant fraction (yield, 15 %). The activity was thermolabile but was resistant to dialysis, freezing, and treatment with SH inhibitors, alkali, charcoal and ion exchangers. Thiamine deficiency did not lower the enzyme activity. Metals were not needed in the reaction mixture though inhibition by chelating agents was recovered with Mg^{++} or Mn^{++}. The enzyme was found to be distributed in the kidney and liver of a rat, dog and hog, and was concentrated in the same $(NH_4)_2SO_4$ fraction.

Among the keto acids tested, substrates were limited to DKG or other 2,3-diketoaldonic acids derived from ascorbic acid homologues. DAA was also decarboxylated but with a lag owing to its hydrolysis into DKG. The reaction products of DKG were absorbed with Dowex-1 column and eluted with formic acid. They were identified as lyxonic acid and probably xylonic acid on paper chromatograms with three solvent systems. Highly reductive spots were also found which were formed nonenzymically. The reaction was not reversed as shown by the use of $^{14}CO_2$.

The fact that two reaction products were obtained by the purified decarboxylation enzyme suggested at least three possibilities:

(1) Epimerization between L-lyxonate and L-xylonate. However, addition of one of these compounds did not produce the other.

(2) Intramolecular oxido-reduction of L-xylosone formed after enzymic decarboxylation. However, neither of the aldonates was formed from xylosone in this reaction mixture.

(3) Enolization of DKG before decarboxylation, which process results in the formation of two true substrates, *i.e. cis-* and *trans*-3,4-enediol-L-gulonic acid. The presence of the enediolform of DKG[6] in neutral solution was suggested by the pH-titration curve and by the reductivity. *Cis-trans* isomerism of enediol compounds has also been reported[7,8]. The disodium salt of DKG was synthesized from L-ascorbic acid by SeO_2 oxidation followed by addition of 2.5 equiv. of NaOH in methanol. The analytical values of the resulting yellow precipitate agreed with the formula of $C_6H_6O_7Na_2 \cdot H_2O$. Rapid decarboxylation was observed without a lag when this

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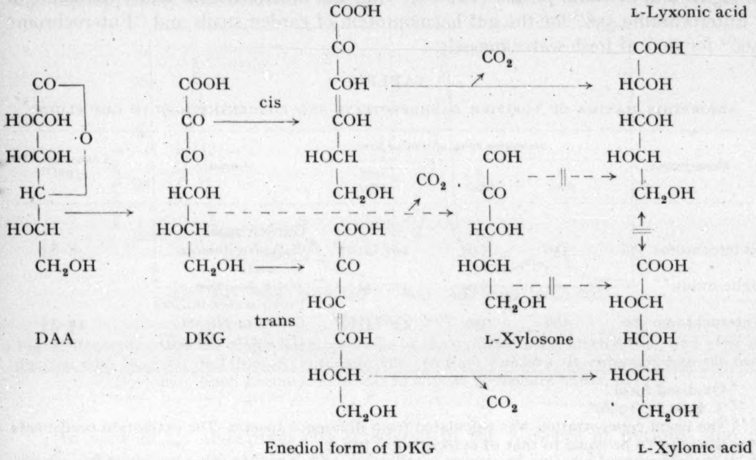
Abbreviations: DAA, dehydro-L-ascorbic acid; DKG, 2,3-diketo-L-gulonic acid; DEAE-, dimethylaminoethyl-.

disodium DKG was used as a substrate, contrary to DKG which showed a 1-min lag. The sharp decrease in activity in acid conditions (pH 6.2) and the high apparent K_m ($1.5 \times 10^{-2}\,M$) also supported the presence of a enediol intermediate.



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Cytochrome-like haemoproteins in the gut fluids of molluscs

Helicorubin, a haemoprotein of unknown physiological function, has been found in the gut fluids of certain molluscs, polychaetes and crustaceans[1–3], and some of its physical and biochemical properties have recently been characterized with purified preparations[4,5]. Except for erythrocruorin and chlorocruorin occurring in the blood of invertebrates, helicorubin is the only haemoprotein found extracellular in high concentrations.

Abbreviation: DEAE, diethylaminoethyl-.

During the studies on cytochromes of invertebrates[6,7], the author discovered two cytochrome-like haemoproteins, one in the gut fluids of garden snails, *Euhadra amaliae* and *E. sandai*, and the other in those of fresh-water mussels, *Hyriopsis schlegelii* and *Cristaria plicata* (Table I). They are described here under the name of "Enterochrome 556" for the gut haemoprotein of garden snails and "Enterochrome 566" for that of fresh-water mussels.

TABLE I

ABSORPTION MAXIMA OF PURIFIED HAEMOPROTEINS AND CONCENTRATIONS IN GUT FLUIDS

Haemoprotein	*Absorption bands of reduced form*			*Animal*	*Concentration**** (*μM*)
	α (*mμ*)	*β* (*mμ*)	*Soret* (*mμ*)		
				Garden snail	
Enterochrome 556	556	526	424 (412)*	*Euhadra amaliae*	58–83
				E. sandai	—
Helicorubin**	561.5	530	427 (415.5)*	*Helix pomatia*	
				Fresh-water mussel	
Enterochrome 566	566	530	430 (416)*	*Cristaria plicata*	14–35
				Hyriopsis schlegelii	18–21

* Oxidized form.
** J. KEILIN (1956)[4].
*** The haem concentration was calculated from difference spectra. The extinction coefficients were assumed to be equal to that of cytochrome *b* (ref. 8).

The collected gut fluid from the gastrointestinal tracts of starved garden snails given only a little water or from the stomach cavities of starved fresh-water mussels, was diluted 3-fold with water, and treated with a small amount of 0.5 *M* lead acetate. After removal of the excess of lead with a small amount of Amberlite CG-50 (sodium form), the solution was fractionated with $(NH_4)_2SO_4$. The fraction precipitating between 0.45 and 0.75 satn., was collected, dialyzed, and then applied on a DEAE-cellulose column equilibrated with 0.01 *M* phosphate buffer, pH 7.0, the elution being carried out with the same buffer. The denatured haemoproteins and brown pigments were strongly absorbed on the top of the column and not elutable by the buffer. The eluted fraction, after saturated with $(NH_4)_2SO_4$ at 0.75, was dialyzed against 0.001 *M* phosphate buffer, pH 7.0, then concentrated by the freeze-drying.

Both haemoprotein preparations thus obtained were clear orange-red solutions. Figs. 1A and B show their visible absorption spectra. Enterochrome 566 had a shoulder at 560 mμ in addition to the α-band at 566 mμ. The prosthetic groups of both enterochromes can be easily split off by treating them with acetone containing 1 % HCl, and the acid haematins thus separated were soluble in ether. The reduced bands of the alkaline pyridine haemochromogen prepared from enterochrome 566 lie at 557 mμ (α), 526 mμ (β) and 418 mμ (Soret). However, those of enterochrome 556 lie at 553 mμ (α), 521 mμ (β) and 415 mμ (Soret). The oxidation–reduction potentials of the two haemoproteins, determined with the aid of 2,6-dichlorophenol-indophenol and toluylene blue at 30°, were found to be nearly equal , *viz.* + 0.14 V at pH 7.8 and + 0.22 V at pH 6.4. Both of the enterochromes migrated toward the cathode when they were subjected to paper electrophoresis (0.05 *M* phosphate buffer, pH 7.0) and in this respect resemble mammalian cytochrome *c*. The enterochrome preparations showed no peroxidase activity when assayed in the guaiacol test[9]. These haemoproteins did not react with CO and cyanide. They were reducible by ascorbate as readily as by $Na_2S_2O_4$, but not reducible by succinate or reduced diphosphopyridine nucleotide.

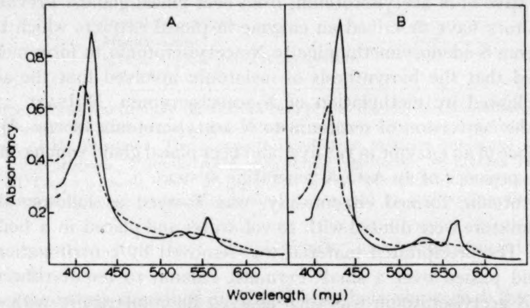

Fig. 1. Absorption spectra of gut haemoproteins in the oxidized form (dotted line) and after reduction with $Na_2S_2O_4$ (full line). A, enterochrome 556 from *Euhadra*; B, enterochrome 566 from *Hyriopsis*. Each haemoprotein was in 0.05 *M* phosphate buffer, pH 7.0.

In view of its absorption characteristics in the presence of pyridine and its ready splitting in acid acetone, the prosthetic group of enterochrome 566 may be regarded as protohaem. The haem moiety of enterochrome 556, on the other hand, seems to represent a new type of haem, in that it is easily split by acid acetone and has an α-peak at 553 mμ in its pyridine haemochromogen. Although enterochrome 556, in certain respects, is similar to cytochrome *h* extracted from the hepatopancreas of *Helix pomatia*[4], it is very different from cytochrome *h* in the following respects: mode of existence, position of the Soret absorption band and behavior on paper electrophoresis.

A full report of this work will be presented for publication shortly. The author wish to thank Prof. RYO SATO for his continual interest and advice.

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Biosynthesis of melatonin: enzymic conversion of serotonin to N-acetylserotonin

Recently LERNER and coworkers[1] isolated melatonin (5-methoxy-N-acetyltryptamine), a derivative of N-acetylserotonin, from beef pineal glands. Previous studies from our laboratory have described an enzyme in pineal extracts which transfers a methyl group from S-adenosylmethionine to N-acetylserotonin to form melatonin[2,3]. It was suggested that the biosynthesis of melatonin involved first the acetylation of serotonin, followed by methylation of N-acetylserotonin. MCISAAC AND PAGE[4] have reported the conversion of serotonin to N-acetylserotonin *in vivo*. We wish to report the presence of an enzyme in rat liver and beef pineal gland which can acetylate serotonin in the presence of an AcCoA-generating system.

N-Acetylserotonin, formed enzymically, was assayed as follows: aliquots of the incubation mixture were diluted with 10 vol. water and placed in a boiling-water bath for 1 min. The precipitated material was removed by centrifugation and the supernatant fluid passed over a small Permutit column to remove the substrate, serotonin. The N-acetylserotonin was then assayed fluorometrically with excitation wavelength, 295 mμ; fluorescence wavelength, 360 mμ.

As shown in Table I a soluble supernatant fraction of rat liver was able to acetylate serotonin in the presence of an AcCoA-generating system. The N-acetylserotonin formed in this manner was further identified by paper chromatography, as well as by enzymic conversion to melatonin with a purified hydroxyindole-O-methyl transferase from beef pineal glands[2,3].

TABLE I

ACETYLATION OF SEROTONIN BY RAT LIVER

	N-acetylserotonin formed (μg/h)
Complete system	5.8
Minus Liver	< 0.3
Minus AcCoA-generating system	0

Livers from rats, pretreated with iproniazid to inhibit monoamine oxidase, were homogenized in 3 vol. of ice-cold water and centrifuged at 78,000 × *g* to obtain a soluble supernatant fraction. The complete incubation system contained 0.3 ml of soluble supernatant, 0.34 μmole serotonin, 50 μmoles Tris buffer, pH 8.0, 15 μmoles EDTA, 0.5 μmole CoA, 12.5 μmoles acetyl phosphate, phosphotransacetylase* (0.04 mg protein), and water to a total volume of 1.5 ml. Incubations were carried out at 37°.

Soluble supernatant fractions obtained from rat brain and beef pineal glands were also shown to acetylate serotonin. However, the serotonin-acetylating system in the brain and pineal gland was less active and more labile than that in the liver. Since beef pineal extracts also contain hydroxyindole-O-methyl transferase it was possible to show the over-all conversion of serotonin to melatonin using a partially

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Abbreviations: CoA, coenzyme A; AcCoA, acetyl-coenzyme A; AMe, S-adenosylmethionine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

* A crude preparation of phosphotransacetylase was kindly supplied by Dr. ROY VAGELOS, National Heart Institute.

TABLE II

FORMATION OF MELATONIN FROM SEROTONIN BY BEEF PINEAL EXTRACTS

	Melatonin formed (*μg/h*)
Complete system	0.7
Minus AMe	0.0
Minus AcCoA-generating system	0.0

A soluble supernatant fraction obtained from beef pineal glands was fractionated with ammonium sulfate and the protein that precipitated between 33–60% satn. was dissolved in water and used as the source of the enzymes. The complete incubation mixture contained ammonium sulfate fraction (9.5 mg protein), 1 μmole AMe, 0.34 μmole serotonin, 50 μmoles Tris buffer, pH 8.0, 15 μmoles EDTA, 0.5 μmole CoA, 12.5 μmoles acetyl phosphate, phosphotransacetylase and water to a total volume of 1.5 ml. Melatonin was assayed by a procedure described elsewhere[5].

purified preparation from beef pineal glands, an AcCoA-generating system and AMe (Table II).

The experiments described here and elsewhere show that the biosynthesis of melatonin proceeds from serotonin as follows:

$$\text{Serotonin} \xrightarrow{\text{AcCoA}} \text{N-acetylserotonin} \xrightarrow{\text{AMe}} \text{Melatonin}$$

Further studies are now in progress on the purification and properties of this enzyme system.

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A comparison of the thermal dissociation of two polynucleotide helices in H_2O and D_2O

In studies of the infrared spectra of nucleic acid derivatives it is desirable to work in aqueous solution, since this eliminates the necessity of assumptions about possible changes in spectra or tautomeric form in going from the usual infrared solvents to the biologically important solvent, water. D_2O has the advantage over water of possessing a window in the region of double bond absorption, and spectra of alkylated model compounds[1], nucleotides[1,2], polynucleotides[1], and of nucleic acids[3] have been studied in this solvent. In the use of D_2O there is the implicit assumption (though a much smaller one than is necessary in applying $CHCl_3$ or CCl_4 spectra to water) that results obtained in D_2O apply to H_2O. To investigate this point the dissociation of hydrogen-bonded polynucleotide* mixtures with increasing temperature (melting curve) was investigated in H_2O and D_2O, both as a matter of intrinsic interest and as background information for the infrared studies.

Melting curves of a number of polynucleotide mixtures have been reported[5,6], either u.v.-absorption or optical rotation being used to follow the thermal dissociation of the helices.

The melting temperatures, T_m, are defined in the present case as the maxima of the derivative curves for poly(A + U) and poly(I + C). This method has the advantage of permitting a characteristic temperature to be determined when very high or low temperatures make measurement of the full curve inconvenient or impossible. (This value of T_m is a few degrees higher then the temperature at which the absorption has had half its maximum increase).

The results, shown in Figs. 1 and 2, show that there is no difference in T_m in D_2O and H_2O within the limits of accuracy of the measurements (the values of T_m can be determined to within about 0.5°; any difference in T_m in D_2O and H_2O is less than 1°). It is concluded from these curves that the chemical properties measured in D_2O apply also to H_2O as a solvent.

Recent experiments have shown differences in melting-out behavior of a polypeptide[7], of ribonuclease[8], and of gelatin[9] in hydrogen and deuterium systems, but these results do not conflict with those cited above showing no difference in T_m in D_2O and H_2O. The relative stability of the helix in the two solvents will depend

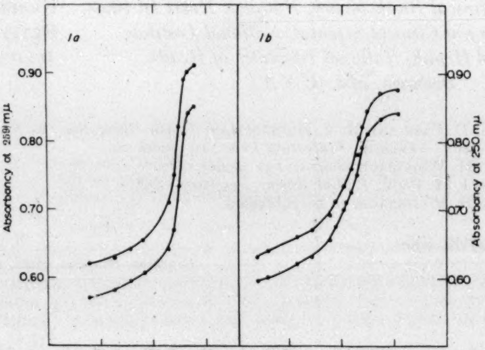

Fig. 1a. Poly A + Poly U, 0.1 *M* NaCl, 0.05 *M* sodium cacodylate, pH 7.2. Upper curve, H_2O; lower curve, D_2O. The temperature at half maximum increase of absorbancy is 59.3° in D_2O and 58.7° in H_2O.

Fig. 1b. Poly I + Poly C, 0.125 *M* NaCl, 0.05 *M* sodium cacodylate, pH 7.0. Upper curve, D_2O; lower curve, H_2O. The temperature at half maximum increase of absorbancy is 53.3° in D_2O and 53.0° in H_2O.

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* The polynucleotides used are the homopolymers polyadenylic acid (Poly A), polyuridylic acid (Poly U), polycytidylic acid (Poly C) and polyinosinic acid (Poly I), prepared with polynucleotide phosphorylase[4].

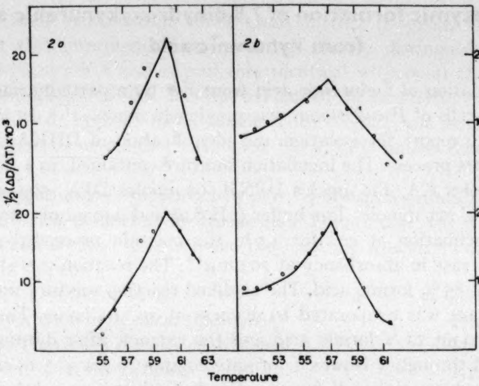

Fig. 2a. Poly A + Poly U; derivative plots of curves in 1a. Upper curve, T_m = 60° ± 0.5° in H_2O; lower curve T_m = 60° ± 0.5° in D_2O.

Fig. 2b. Poly I + Poly C; derivative plots of curves in Fig. 1b. Upper curve, T_m = 57 ± 0.5° inD_2O; lower curve, T_m = 57° ± 0.5° in H_2O.

primarily on whether the quantity (*Fp–p—Fp–s*)* is greater than, equal to, or less than the same quantity in D_2O. With the polynucleotides studied here the values must be very nearly the same in the two solvents**.

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** Dr. W. F. HARRINGTON has pointed out to the author the possibility that the ideas presented by GALLAGHER[10] on the effect of isotope substitution on hydrogen-bond length and strength may permit a prediction of the direction and possibly of magnitude of deuterium substitution on the melting temperature of polymers. At the present time, however, the necessary bond distances and the shape of GALLAGHER's curve (Fig. 1)[10] above 2.8 Å are probably not known with sufficient accuracy to make the prediction reliable in the case of the polynucleotides.

* Where *Fp-p* is the energy of the hydrogen bonds from one polymer chain to another in the helix and *Fp-s* is the energy of the hydrogen bonds from solvent to the polymer chains after they have dissociated.

The enzymic formation of 7,8-dihydroxykynurenic acid from kynurenic acid

The enzymic formation of L-glutamic acid from KA by a partially purified enzyme preparation from cells of *Pseudomonas* was previously reported[1,2]. In this communication, we wish to report the isolation and identification of DHKA, a presumable intermediate in this process. The incubation mixture contained, in a final volume of 3,150 ml, 240 μmoles KA, 120 μmoles DPNH, 90 μmoles DPN, 500 μmoles ferrous ammonium sulfate, 225 mmoles Tris buffer (pH 8.0) and 240 ml of enzyme[*] (960 mg protein). After incubation at 20° for 3.5 h, the reaction proceeded over 80 % as judged by the increase in absorbancy at 293 mμ[**]. The reaction was stopped by the addition of 600 ml 85 % formic acid. The acidified reaction mixture was centrifuged and the supernatant was evaporated to dryness at 40° *in vacuo*. The residue was extracted with 130 ml 22 *N* formic acid and the extract, after dilution with 7 vol. water, was passed through a Dowex 1 formate column (6 cm × 3.14 cm^2, 8 % cross linkage). After washing with 4 *N* formic acid, the column was eluted successively with 6 *N*, 12 *N* and 22 *N* formic acid. The combined eluates were concentrated to dryness *in vacuo*. DHKA, obtained as a light yellow crystal (21 mg, 85 μmoles) was recrystallized several times from 47.5 % HBr. It was identified by m.p. (both authentic[***] and isolated materials started to darken at 275° and almost blacken at 290° without melting), analysis (Found: C, 40.24; H, 2.87. Calc. for $C_{10}H_7NO_5 \cdot HBr$: C, 39.76; H, 2.67), the u.v. spectrum[§], the infrared spectrum and by paper electrophoresis.

Concurrently, DHKA was synthesized by BEHRMAN AND TANAKA and was found to be rapidly converted to a yellow, as yet unidentified compound by a similar bacterial extract[3]. We have previously reported[4] enzymic synthesis of DHKA starting from 3,4-dihydroxykynurenine[5] by *Pseudomonas* kynurenine transaminase in the presence of α-ketoglutarate and obtained similar results. The present communication provides definitive proof that DHKA is a metabolite of KA by the *Pseudomonas* enzyme.

When a partially purified enzyme preparation was incubated with KA in the presence of reduced pyridine nucleotides (DPNH or TPNH) and oxygen, the accumulation of a compound with λ_{max} at 314 mμ at pH 8.0, was observed. This compound was then dehydrogenated to form DHKA by a DPN-linked dehydrogenase.

Abbreviations: KA, kynurenic acid; DHKA, 7,8-dihydroxykynurenic acid; DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane.

[*] The supernatant fraction, which was prepared as previously described[1], was fractionated with ammonium sulfate in the presence of 0.01 *M* cysteine. The precipitate obtained between 40 and 50% satn. was collected by centrifugation and dissolved in an amount of 0.02 *M* Tris buffer, pH 8.0, containing 0.01 *M* cysteine, equal to the volume of the original enzyme solution. The enzyme was again fractionated with ammonium sulfate and the precipitate obtained with 50% satn. was dissolved in the same buffer.

[**] DHKA shows a characteristic u.v. spectrum (λ_{max}, 293 mμ, ε, 18,000) in the presence of excess Fe^{++} and Tris buffer, pH 8.0.

[***] An authentic sample of DHKA was synthesized by the reduction and demethylation of ethyl-β-(2-nitro-3,4-dimethoxybenzoyl-)pyruvate (T. TOKUYAMA, S. SENOH AND T. SAKAN, unpublished procedure).

[§] Isolated material shows three maxima in 3 *N* HCl; ε_{266}, 34,400; ε_{325}, 5,200; ε_{386}, 2,300. Those of an authentic sample are ε_{266}, 37,200; ε_{325}, 5460; ε_{386}, 2,410.

Although the final identification must await further progress, available evidence indicates that this intermediate compound is clearly distinguishable from either 7- or 8-monohydroxy-KA and is probably identical with or at the same oxidation level as 7,8-dihydrodiol of KA. The mechanism of the conversion of this compound to DHKA, therefore, appears to be analogous to the enzymic formation of catechol from 3,5-cyclohexadiene-1,2-diol catalyzed by a TPN-linked dehydrogenase from rabbit liver[6].

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Enzymic formation of acetyl-CoA and CO_2 from glutaryl-CoA

Glutaric acid has been shown to be an intermediate metabolite of lysine degradation[1], and recently to be a product of tryptophan metabolism in the rat[2]. Although several investigators have described the conversion *in vivo* of glutaric acid to acetic acid[2,3], the exact pathway of glutaric acid metabolism is yet to be elucidated. In this communication, we wish to report that 1 mole of glutaric acid is converted to 2 moles of acetate and 1 mole of CO_2 by a partially purified enzyme preparation from *Pseudomonas*, and glutaryl-CoA is proposed to be an intermediate in this process.

Pseudomonas fluorescens (ATCC 11299) was grown as described previously[4], except that 0.1 % glutarate and 0.5 % $(NH_4)_2SO_4$ were used as major carbon and nitrogen sources, respectively. Cell-free extracts were prepared by extracting 5 g of acetone-dried cells with 50 ml 0.02 *M* potassium phosphate buffer, pH 6.8, for 20 min at 0°, followed by centrifugation at 20,000 × *g* for 30 min. The supernatant fraction thus obtained was treated with protamine sulfate.

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The reaction mixture (1.0 ml) containing 100 μmoles potassium glutarate, 10 μmoles ATP, 0.5 μmole CoA, 10 μmoles of reduced glutathione, 5 μmoles of $MgCl_2$, 1,000 μmoles of salt-free NH_2OH, 100 μmoles phosphate buffer, pH 6.3, and 30 mg of the enzyme preparation which had been treated with both Dowex 1 and charcoal, was incubated at 35° for 60 min. 6.5 μmoles of a hydroxamate derivative were produced[5], which was tentatively identified as glutaromonohydroxamate by paper chromatography using four different solvent systems* and high-voltage paper electrophoresis**. In the absence of ATP or CoA, practically no glutaromonohydroxamate was produced.

When GP was incubated with the enzyme preparation under aerobic conditions, approximately 2 moles acetate and 1 mole CO_2 were produced for each mole of GP utilized (Table I). When [1,5-^{14}C]GP was employed as a substrate, total radioactivity of CO_2 was almost equal to that of acetate and the specific activity of acetate was approximately one fourth of that of the original substrate. With [3-^{14}C]GP***, however, CO_2 evolved contained essentially no radioactivity but the specific activity of acetate was diluted approximately 2-fold. When neutral NH_2OH was added after the incubation at a final concentration of 0.5 *M*, a new hydroxamate was formed which was identified as acetohydroxamate by paper chromatography and

TABLE I

STOICHIOMETRY OF THE OVERALL REACTION

The reaction mixture contained 15 μmoles [1,5-^{14}C]GP (5,800 counts/min/μmole) or [3-^{14}C]GP (5,790 counts/min/μmole), 50 μmoles of phosphate buffer, pH 6.8, and 10 mg of the enzyme preparation in a final volume of 1.0 ml. After incubation was carried out for 60 min at 35°, the reaction was stopped by the addition of 0.2 ml of 2 *N* KOH, and the mixture was heated for 1 min in a boiling-water bath in order to hydrolyze the thiol esters. Then the mixture was acidified by the addition of 0.5 ml 2 *N* H_2SO_4, and CO_2 evolved was trapped in alkali, An aliquot of the remaining solution was chromatographed on a silicic acid column (20 × 1 cm) and was eluted with a chloroform–*n*-butanol mixture. 2-ml fractions were collected, and titrated with 0.002 *N* KOH. Radioactivity was measured with an aliquot of each fraction.

Substrate	*Gas phase*	*Δ Glutarate*		*Δ CO_2*		*Δ Acetate*		
		Total (counts/min)	*Titration (μmoles)*	*Total (counts/min)*	*Δ* (μmoles)*	*Total (counts/min)*	*Titration (μmoles)*	*Specific activity (counts/min/μmole)*
[1,5-^{14}C]Glutaryl-pantheteine	air	—12,400	—2.0	+5,660	+1.9	+ 5,520	+3.5	1,620
	N_2	—400	—0.2	+20	—	+20	0.2	—
[3-^{14}C]Glutaryl-pantheteine	air	—11,100	—2.0	+70	—	+10,210	+3.6	2,740
	N_2	—500	—0.2	0	—	+30	0.2	—

* Calculated from radioactivity.

Abbreviations: CoA, coenzyme A; GP, glutarylpantetheine; ATP, adenosine triphosphate.

* Paper chromatography was carried out on Whatman No. 1 paper. Spots were visualized by spraying with an acidic $FeCl_3$ solution. R_F values of hydroxamates of glutarate and acetate with xylol–phenol–formic acid (5:5:2) were 0.46 and 0.62; isobutyric acid–*n*-butanol–water (2:2:1), 0.40 and 0.50; with *n*-propanol–ammonia (3:2), 0.12 and 0.33; with ethanol–ammonia–water (20:1:4), 0.40 and 0.54, respectively.

** High-voltage paper electrophoresis was carried out on Whatman No. 3 paper (15 × 55 cm; pyridine–acetic acid buffer, pH 6.5[6]; coolant, *n*-hexane; 2,000 V; 40 min). Mobilities of glutaromono- and acetohydroxamate were 8.0 cm to the anode and 1.2 cm to the cathode, respectively.

*** The authors are indebted to Dr. R. E. KOEPPE for a generous sample of [3-^{14}C]glutaric acid.

high-voltage paper electrophoresis. Acetohydroxamate thus obtained from either [1,5-^{14}C]GP or [3-^{14}C]GP contained radioactivity. However, when free [^{14}C]acetate was incubated together with cold GP under the same conditions, no radioactivity was incorporated into the acetohydroxamate isolated. The radioactive acetate formed from either [1,5-^{14}C]GP or [3-^{14}C]GP contained almost all radioactivity in the carboxyl carbon[7]. These results indicate that free carboxyl carbon (C-5) of GP was converted to CO_2, and both C-3,4 and C-1,2 of GP were converted to C-1,2 of acetylpantheteine respectively. Under anaerobic conditions, essentially no acetate or CO_2 was produced, but oxygen could be replaced by pyocyanine and 2,3,5-triphenyltetrazolium chloride. Radioactive acetate enzymically formed was identified by paper chromatography using three different solvent systems*, and by partition chromatography on a silicic acid column[9]. Further evidence for the identity was provided by the constant specific activity upon recrystallization of its *p*-bromophenacyl ester[10].

Similar results were obtained with glutaryl-CoA, and acetyl-CoA enzymically formed was identified with *p*-nitroaniline-acetylating enzyme from pigeon liver[11]. Further studies are currently in progress in order to elucidate the intermediates from glutaryl-CoA to acetyl-CoA**.

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* Paper chromatography was carried out on Whatman No. 1 paper. Spots were visualized by spraying with reduced ninhydrin[8]. R_F value of acetate with ethanol–ammonia–water (20:1:4) was 0.52; with *n*-propanol–ammonia (3:2), 0.66 and with tetrahydrofuran–ammonia–water (15:2:3), 0.62.

** Recently it was reported that glutaryl-CoA was isomerized to ethylmalonyl-CoA, followed by decarboxylation to butyryl-CoA in animal tissues[12,13], but this isomerization of glutaryl-CoA was not observed in our bacterial system. Neither butyryl-CoA nor succinyl-CoA was converted to acetyl-CoA under the above conditions.

Biosynthesis of S-methylmethionine in the jack bean

McRorie *et al.*[1] showed S-methylmethionine to be a natural constituent of several plant tissues. Several groups of workers have studied the metabolism of S-methylmethionine in animals[2], microorganisms[3] and plants[4].

We have shown that S-methylmethionine is a major metabolite of methionine in young jack-bean seedlings[5] and the present study demonstrates its synthesis from methionine and S-adenosylmethionine in cell-free extracts of jack-bean roots. The reaction presumably involves the transfer of a methyl group from S-adenosylmethionine to the sulfur of methionine as shown in the following equation:

$$\text{Methionine} + \text{S-adenosylmethionine} \rightleftharpoons \text{S-methylmethionine} + (\text{S-adenosylhomocysteine})$$

When jack-bean seedlings are grown in the presence of labeled methionine 40–80% of the non-protein radioactivity is found in a single basic amino acid. This amino acid has been isolated and identified as S-methylmethionine.

S-methylmethionine was isolated from plant extracts by a method, which will be described in a later paper, involving chromatography on the ammonium forms of Dowex 50 and Amberlite CG 120. The radioactive compound isolated from plants grown on L-[^{35}S]methionine or DL-[2-^{14}C]methionine was shown to be S-methylmethionine by comparison with authentic material in several chromatography solvents including the two-dimensional system of Calvin and Benson[6], and by identification of the radioactive products of degradative procedures (Table I). When the roots and the remainder of the plant were extracted separately, it was found that the labeled S-methylmethionine was almost entirely restricted to the roots (Table II).

Because of the localization of S-methylmethionine in the roots of intact seedlings, root tissue was used as a source of enzyme in the study of S-methylmethionine synthesis.

Cell-free extracts were prepared by grinding jack-bean hypocotyls or roots of young seedlings with 2.5 parts of buffer (0.1 *M* potassium phosphate, pH 7.2, 0.008 *M*

TABLE I

Treatment	*Labeled product*	*Identification procedure*
6 *N* HCl, 100°, 7 h	[^{14}C]methionine	Paper chromatography
1 *N* NaOH, 100°, 3 h	[^{14}C]homoserine	Paper chromatography
KBH_4	[^{14}C]aminobutyric acid	Paper chromatography
10 *N* KOH, 100°, 20 min	[^{35}S]dimethyl sulfide	Gas chromatography

TABLE II

Green plant extract (without roots)	*Root extract*		
Total counts/min	*Total counts/min*	*% Activity in basic amino acid fraction*	*Basic amino acid activity in S-methylmethionine peak of Amberlite CG-120*
$2.46 \cdot 10^4$	$1.92 \cdot 10^5$	83.3	—
—	$2.12 \cdot 10^6$	39.4	97.5

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glutathione, 30% polyethylene glycol with the omissions noted in Table III) and spinning the homogenate in a Lourdes Refrigerated Centrifuge. The supernatant solution was incubated with labeled methionine under the conditions described in Table III. The basic amino acids were isolated from the deproteinized incubation mixtures by chromatography on hydrogen Dowex 50 using HCl as the eluant.

TABLE III

Homogenized and incubation medium	*% Isotope incorporated into S-methylmethionine*
Complete	5.13
— S-Adenosylmethionine	0.62
Complete	4.80
— Glutathione	1.49
Complete	7.22
— Polyethylene glycol	0.74

Complete medium contains 0.07 *M* potassium phosphate, pH 7.2, 0.006 *M* glutathione, 21% polyethylene glycol-400 (Fisher), 0.0006 *M* S-adenosylmethionine, 0.0002 *M* methionine. Each incubation vessel has a total volume of 3.3 ml and contains 1 ml root extract and 2–5·10^5 counts/min [^{14}C-*Me*]methionine. Vessels were incubated at 30° for 4 h.

S-methylmethionine was shown to be the only radioactive compound in this fraction by paper chromatography and electrophoresis and by chromatography on ammonium Amberlite CG 120 (Table II). The requirements for S-methylmethionine synthesis in a cell-free system are given in Table III. The requirement for S-adenosylmethionine at low concentration is thought to indicate the participation of this substance as a reactant as depicted in the equation above. Inclusion of polyethylene glycol and glutathione in the homogenization and incubation medium yields greater activity. The function of polyethylene glycol is not clear, but similar polymers have been shown to stabilize aldolase and triose phosphate dehydrogenase in crude plant extracts[7]. Incorporation of variously labeled methionine into S-methylmethionine by cell-free extracts is shown in Table IV. The observation that [^{35}S]- and [^{14}C-*Me*]methionine, which both have the L-configuration, are incorporated to roughly twice the extent of DL-[2-^{14}C]methionine suggests that this enzyme is specific for L-methionine.

These observations represent the first example of the biosynthesis of a naturally occurring sulfonium compound other than S-adenosylmethionine; should studies with more highly purified enzyme preparations demonstrate reversibility, this reaction could permit S-methylmethionine to function as a biological reservoir of active methyl groups.

TABLE IV

Labeled compound	*% Incorporation*
L-[^{35}S]Methionine	10.3
DL-[2-^{14}C]Methionine	4.1
L-[^{14}C-*Me*]Methionine	8.0

Conditions similar to Table III except each vessel contained 3 ml root extract. Incubation 5 h at 30°.

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Transfer of radioactivity from the prelabelled cell debris to the microsomal protein

The study of HENDLER[1], showing that mince of hen oviduct incorporates [^{14}C]amino acids into protein but homogenates of the same tissue do not incorporate the amino acids, suggested to us the possibility of the involvement of a factor similar to the prelabelled cell debris concerned in the incorporation of [^{14}C]amino acids into protein in silkworms' silk glands.

In fact, as shown in our previous paper[2], the ability to incorporate [1-^{14}C]glycine into the protein fraction of silk glands was decreased to about one hundredth by the homogenation of the glands.

Studies carried out using subcellular fractionation and incubation of silk glands essentially according to the methods of SHIMURA[3] for the incorporation of ^{14}C-labelled precursors into microsomal protein, with cell debris as the prelabelled source of ^{14}C, showed a marked increase in the radioactivity incorporated into protein. When [1-^{14}C]glycine was used as the source of radioactivity, the relative specific activity of the microsomal protein was less than 0.0001 %, whereas in the case of cell debris as precursors the value was generally around 20 %.

The complete reaction mixture contained approximately 5.8 mg E_1 protein, 7.8 mg E_2 protein and 3.2 mg E_3 protein; 16 μmoles ATP; 20 μmoles phosphocreatin; 8 μmoles $MgCl_2$; 120 μmoles KCl; 100 μmoles Tris–HCl (pH 8.0); 800 μmoles sucrose; 1.0 mg creatine kinase; 0.5 μmole GTP; and 19 amino acids (8 μmoles DL-alanine, 4 μmoles L-serine, 0.8 μmole L-tyrosine, 2 μmoles of DL-valine and DL-threonine, 1 μmole of L-aspartic acid, L-asparagine, L-phenylalanine and L-glutamic acid, 0.5 μmole of L-isoleucine, L-histidine, L-leucine, L-proline and L-arginine, 0.25 μmole of L-glutamine, L-cystine, L-methionine, L-tryptophan and L-lysine).

[2-^{14}C]Glycine-labelled silk glands cell debris was obtained by the following method: 15 min after the injection of 2 μC [2-^{14}C]glycine *per capita*, the silk glands

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Abbreviations: ATP, adenosine triphosphate; GTP, guanosine triphosphate; Tris, Tris(hydroxymethyl)aminomethane.

TABLE I

TRANSFER OF RADIOACTIVITY FROM [^{14}C]CELL DEBRIS TO MICROSOMAL PROTEIN

System	*Incubation time (min)*	*Radioactivity transferred to microsomal protein counts/min/mg*
Complete system	0	23
Complete system	15	130
Complete system	30	114
Complete system	60	119
Complete system–E_3	30	146 (120*)
Complete system–Amino acids	30	53
Complete system–GTP	30	96
Complete system–ATP and ATP-regenerating system	30	55
Complete system–E_1–E_2–E_3	30	(29**)

Cell debris: The precipitate of the centrifugation of silk-glands homogenate at 700 × *g* for 10 min. E_1 (microsomes): After removal of large particles by centrifugation at 14,000 × *g* for 30 min, the supernatant was acidified at pH 6.1 and recentrifuged at 8500 × *g* for 8 min. Sediment used. E_2 (incorporation enzymes): The supernatant of E_1 was adjusted at pH 4.9 and centrifuged at 8500 × *g* for 8min. Sediment used. E_3 (amino-acid-activating enzymes): The supernatant of E_2.

* Corrected for difference of protein concentration.

** Radioactivity contained in the supernatant after centrifugating at 1400 × *g* for 10 min.

were removed, and the cell-debris fraction was separated by the method of SHIMURA[3]. After thorough washings with 10 % non-labelled glycine solution, the cell debris was twice homogenized in a Waring blender and centrifuged at 700 × *g* for 10 min. Then the pellet was rehomogenized with a Potter-Elvehjem-type homogenizer. Freezing and thawing was carried out in order to destroy any contaminating intact cells. Microscopic examination showed that the major component was nuclear debris without any intact cells. The specific activity was 547 counts/min/mg and a total of 43,000 counts/min was added to each vessel. The final volume of reaction mixtures was 2 ml. After adjusting the pH to 7.4–7.6, the mixtures were incubated at 37° under air and before the reaction was stopped by the addition of 4 ml 8 % $HClO_4$, the incubated media were mixed with 2 ml of non-labelled 10 % glycine solution to eliminate contamination from the surface of the denatured protein[3], and the ^{14}C-labelled cell debris was centrifuged off at 6000 × *g* for 10 min. Contamination remaining in the supernatant fraction was less than 0.5 %. The microsomal fraction was obtained by centrifugation at 8500 × *g* for 8 min after acidification of reaction medium at pH 5.5 Further fractionation was carried out according to the method of HUGGINS AND COHN[4] to remove the phosphatidopeptide from the protein fraction. The radioactivity was measured in a windowless gas-flow counter. All results were expressed in counts/min/mg (ref. weight, 10 mg) according to the study of HENDLER[5].

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Electron-transport and phosphorylation inhibitor in *Tetrahymena* and evidence for its formation by a phospholipase

Preparations of *T. pyriformis* were reported to form by enzymic action a heat-stable agent (or agents) which inhibits various respiratory enzymes of rat-liver mitochondria and *Tetrahymena* homogenate[1]. It has now been shown that this agent inhibits oxidative phosphorylation, activates latent ATPase, and promotes swelling of rat-liver mitochondria. In addition, evidence implicating a phospholipase in its production has been obtained.

Particle preparations (made from homogenates of the GL strain), aged 24 h at 0° and then boiled to destroy the enzyme which produces the inhibitor, uncouple phosphorylation and depress O_2 uptake in the presence of succinate (Table I). Usually, preparations containing 30–50 μg N inhibit phosphorylation 100 % and respiration 55–80 %. Particle preparations, boiled when fresh to reduce inhibitor formation, also suppress phosphorylation and decrease O_2 uptake but much larger amounts are required (Table I). Crystalline bovine serum albumin prevents the effect of the GL

TABLE I

INHIBITION OF OXIDATIVE PHOSPHORYLATION AND OXYGEN UPTAKE OF LIVER MITOCHONDRIA BY A *T. pyriformis* PREPARATION AND PROTECTION BY ALBUMIN

The reaction mixture contained: 40 μmoles potassium phosphate buffer, pH 7.4; 20 μmoles $MgCl_2$; 5 μmoles ATP; 60 μmoles glucose; 140 K.M. units hexokinase; 100 μmoles sodium succinate; 0.042 μmole cytochrome *c*; and 0.28 mg rat-liver mitochondrial N in 63 μmoles sucrose; final volume, 2.8 ml; 0.2 ml 20% KOH in centre well; temp. 30°; duration of experiment 15 min plus 7 min equilibration; phosphate uptake assayed by change in inorganic P by method of Lowry and Lopez[2]. 7 day-old culture of *T. pyriformis* GL homogenized as described[1]; a portion was immediately boiled 20 min; both preparations were stored 24 h at 0° and the unboiled portion was then boiled 20 min. Both preparations were centrifuged 30 min at 105,000 × *g* to obtain the particles (1.1 mg N/ml).

Additions	*ΔO µatoms*	*ΔP µmoles*	*P/O*
None	6.0	10.8	1.8
0.025 ml GL particles, aged (0°) and boiled	4.9	6.5	1.3
0.05 ml GL particles, aged (0°) and boiled	3.1	0.5	0.2
0.1 ml GL particles, aged (0°) and boiled	2.1	0.5	0.2
0.2 ml GL particles, aged (0°) and boiled	0.6	0	0
0.05 ml GL particles, boiled and aged (0°)	6.8	11.0	1.6
0.1 ml GL particles, boiled and aged (0°)	6.1	8.6	1.4
0.2 ml GL particles, boiled and aged (0°)	4.1	3.3	0.8
0.3 ml GL particles, boiled and aged (0°)	3.9	0	0
0.05 ml GL particles, aged (0°) and boiled + 5 mg crystalline bovine albumin	6.8	10.9	1.6

Abbreviations: ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane.

particles on phosphorylation and oxidation. Albumin likewise prevents inhibition of *Tetrahymena* succinic oxidase by the particles. Recently, EDWIN AND GREEN[3] also reported that albumin—and α-tocopherol or ubiquinone—reverses the inhibition of liver-homogenate succinic oxidase induced by a *Tetrahymena*-inhibitor preparation. In other experiments, it was found that the inhibitor completely uncouples phosphorylation linked to α-ketoglutarate or β-hydroxybutyrate oxidation; however, O_2 uptake by the latter system is not decreased even with relatively high levels of the inhibitor preparation.

Aged and boiled GL particles activate the Mg-stimulated latent ATPase activity of liver mitochondria made in 0.25 *M* sucrose and assayed at 30° for 10 min (0.025 *M* Tris, pH 7.5). With higher levels of particles, activity is decreased, and with still higher levels, it is again increased. Relatively large amounts of particle preparations, boiled when fresh, also activate ATPase. ATPase activity of mitochondria treated to annul the latency, *i.e.*, suspended in water and pre-incubated for 15 min at 30°, is not enhanced by the GL preparations. ATPase activity of particles obtained from *T. pyriformis* S or GL in 0.25 *M* sucrose is not latent, does not respond to dinitrophenol, and is not activated by the GL preparations.

In experiments to be described elsewhere, aged and boiled GL particles have been found to promote the swelling of liver mitochondria suspended in 0.125 *M* KCl or 0.25 *M* sucrose and 0.02 *M* Tris, pH 7.4, while preparations which are boiled immediately are not as effective.

The data in Table II show that incubation of "inactive" GL homogenate with crotoxin, a crystalline lecithinase A from *Crotalus t. terrificus*, followed by prolonged boiling to destroy the lecithinase, results in a mixture which inhibits the succinic oxidase of *Tetrahymena* (and liver) almost as markedly as does the active GL homogenate. Purified ribonuclease, deoxyribonuclease, hyaluronidase, trypsin, chymotrypsin, papain, ficin, or wheat-germ lipase do not produce inhibitory substance(s) on incubation with "inactive" GL homogenate. In addition, with crude soy-bean

TABLE II

EFFECT OF LECITHINASE A (CROTOXIN) ON FORMATION OF INHIBITORY SUBSTANCE IN BOILED *T. pyriformis* PREPARATION

1 ml GL homogenate (1.27 mg N), either boiled 30 min when fresh and then aged 7 days at 0° ("inactive") or aged 7 days at 0° (active), was mixed with 0.5 ml water in (1) and (2), respectively, with 0.5 ml lecithinase (20 μg) in (3), and with 0.5 ml lecithinase previously boiled 1 h in (4); in (5), 1 ml water was mixed with 0.5 ml lecithinase. All mixtures were incubated in glass homogenizers 2h at 37°, boiled 1 h, and thoroughly homogenized. 0.5 ml of each mixture was added in duplicate to Warburg vessels containing 150 μmoles sodium succinate, 100 μmoles potassium phosphate buffer, pH 7.1, and *Tetrahymena* S homogenate (about 1 mg N); final volume, 2.8 ml; 0.2 ml 20% KOH in centre well; temp. 30°. Results are given for the first 10 min and for the first h after closing taps and are expressed as % inhibition as compared to control vessels without additions.

No.	*Additions to succinic oxidase system of* T. pyriformis *homogenate*	*Inhibition (%)*	
		10 min	*60 min*
1	Active Gl homogenate	93	95
2	"Inactive" GL homogenate	15	18
3	"Inactive" GL homogenate + lecithinase	63	75
4	"Inactive" GL homogenate + boiled lecithinase	22	35
5	Lecithinase	0	0

lecithin as substrate, the presence of a lecithinase (presumably a phospholipase A) has been demonstrated in homogenates of the GL, S, and W strains of *Tetrahymena*.

These results suggest that the *Tetrahymena* agent(s) which inhibits electron transport, uncouples phosphorylation, and stimulates latent ATPase of rat-liver mitochondria is a fatty acid(s) (and possibly a lysophosphatide) originating from the breakdown of particulate phospholipid(s) by the protozoan phospholipase. A comparison of the patterns of inhibition of various liver and *Tetrahymena* respiratory enzymes produced by oleic acid, lysolecithin, and the protozoan preparation, as well as other studies—all of which will be detailed elsewhere—supports this view. Thus it appears that the inhibitor(s) from *Tetrahymena* is similar to the uncoupling agents extracted by organic solvents from the mitochondria of blow-fly thoraces[4], wax-moth larvae[5], and rat liver[6], and from mitochrome[7].

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The enzymic degradation of heparitin sulfate

Extracts of flavobacteria had been found to degrade various mucopolysaccharides[1]. When the organisms were adapted to either heparin or heparitin sulfate, the "induced enzyme" degraded both substrates[2]. Heparitin sulfate resembles heparin in optical rotation and composition but has only one sulfate group per disaccharide unit. Approximately one-half of the hexosamine units are N-sulfated, the other half are N-acetylated[2].

It has been observed in all cases where "induced enzyme" was used that a loss of carbazole reaction for uronic acid accompanied the hydrolysis. In the case of hyaluronic acid and the chondroitin sulfates, this was shown to be due to the formation of an α-keto acid[3].

When heparitin sulfate was degraded by "induced enzyme", paper chromatography showed the presence of four major products (Fig. 1). The fastest moving component appeared to be N-acetylglucosamine. The three other components, Compounds I, II and III (Fig. 1) were isolated from a cellulose column[4] using butanol–acetic acid–water (50:12:25) as eluant. From 650 mg of a digest, 100 mg N-acetylglucosamine, 200 mg Compound I, 50 mg Compound II and 120 mg Compound III were obtained. None of these compounds gave a significant carbazole reaction for uronic acid. On paper chromatograms, they could be detected by $AgNO_3$[5] but not by Ehrlich's[6] or ninhydrin[7] reagents.

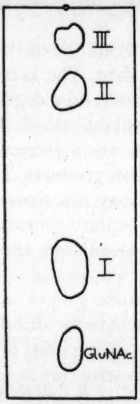

Fig. 1. Reproduction of a chromatogram of a heparitin sulfate digest. Solvent: butanol–acetic acid–water (50:12:25). GluNAc corresponds to N-acetylglucosamine.

Compound I: This material gave the semicarbazide and O-phenylenediamine reactions[3] for α-keto acids. The semicarbazone had the same ultraviolet spectrum as the semicarbazone of the α-keto acid derived from hyaluronate[3]. On paper chromatography in 2 solvent systems, the compound of Spot I had the same R_F value as the α-keto acid derived from hyaluronate.

Compound II: This compound migrated at the same rate as authentic[1] 2-deoxy-2-sulfoamino-D-glucose[8] in 2 solvent systems and on paper electrophoresis in borate buffer. The compound isolated as the barium salt gave the following analyses: hexosamine[9], 47 %; sulfate[9], 23 %, molar ratio sulfate to hexosamine, 1.1. After this compound had been heated to 100° for 1.5 h in 0.04 *N* HCl, paper chromatography showed that it was largely converted to glucosamine.

Compound III: This is also the slowest moving component in a butanol–ethanol–water solvent. However, when cetylpyridinium chloride was added to this solvent, a reversal of rates resulted with Compound III migrating more rapidly than Compound II indicating a higher negative charge[10]. The compound isolated as the barium salt gave the following analyses: heoxsamine, 29 %; sulfate, 33 %, molar ratio of sulfate to hexosamine 2.1. When this compound was heated at 100° for 1.5 h in 0.04 *N* HCl and chromatographed, a new spot, migrating slightly faster than Compound II, appeared. This spot could be detected by $AgNO_3$, ninhydrin or Ehrlich's reagents. A small amount of material corresponding to this new spot was isolated from a cellulose column and gave the following analyses: hexosamine (by ninhydrin[11]), 40 %; sulfate, 21 %, molar ratio sulfate to hexosamine 1.0. These data indicate that this compound is a glucosamine O-sulfate. Compound III was incubated with a glycosulfatase from marine snails (presumably Littorina). When the digest was chromatographed, a spot with the same R_F value in 2 solvents as Compound II (*i.e.*, glucosamine N-sulfate) was detected.

These data indicate that Compound III is a glucosamine disulfate with one of the sulfates on the amino group and the other esterified with one of the available hydroxyls.

Some interesting conclusions about the structure and degradation of heparitin sulfate can be drawn from these data. The breakdown mechanism appears to be similar to that of other mucopolysaccharides degraded by "induced" flavobacterium enzyme, proceeding via unsaturated uronides[3]. Uronic acid color reaction is lost concomitant with hydrolysis, and acetylglucosamine, sulfated hexosamines and α-keto acid are the major degradation products. The presence of a disulfated glucosamine as a major product shows that the structure of heparitin sulfate is closely related to heparin. Since heparitin sulfate contains about 16 % sulfate and most of the N-sulfated glucosamine is also O-sulfated, the N-acetylated glucosamine moiety must be mainly sulfate-free.

A tentative structure of heparitin sulfate would be a polymer with a section containing repeating units of D-glucosamine disulfate and D-glucuronic acid. To this a sulfate-free portion, containing repeating units of N-acetylglucosamine and D-glucuronic acid, would be attached. This structure is also supported by studies on N-desulfated heparitin sulfate[2] which are in progress. When the N-desulfated compound (containing about 7 % sulfate) was incubated with the flavobacterium enzyme, the increase in reducing value was about one-half of that obtained with heparitin sulfate itself. When this digest was dialyzed, the non-dialyzable portion (about 40 % of the total) had a sulfate content of 15 %. A chromatogram of the dialyzable portion showed the presence of Compound I and acetylglucosamine only. The enzyme, apparently unable to hydrolyze the bonds at the free amino positions, had split the N-acetylated moieties, leaving a core containing repeating units of uronic acid and glucosamine O-sulfate. This finding, suggests the possibility of a branched structure of heparitin sulfate.

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CHANGES IN LEVELS OF TRIPHOSPHOPYRIDINE NUCLEOTIDE IN MARINE EGGS SUBSEQUENT TO FERTILIZATION

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SUMMARY

1. The steady-state levels of oxidized and reduced TPN have been measured in two species of marine eggs, *Arbacia punctulata* and *Spisula solidissima*, before and after fertilization.

2. Within 1 to 3 h after fertilization a 3 to 7 fold increase in concentrations of TPNH was observed, but no significant change in concentrations of oxidized TPN.

3. The net synthesis of TPN is discussed in relation to the predominant role of the hexose monophosphate shunt in glucose utilization by these eggs.

INTRODUCTION

It has been shown that during the early stages of cleavage of eggs of *Arbacia punctulata* utilization of glucose is predominantly via the hexose monophosphate shunt[1,2]. As development proceeds, the glycolytic pathway becomes quantitatively more important. However the amount of glucose oxidized via pathways requiring TPN increases many fold as development continues. The activities of two enzymes concerned with oxidation of glucose-6-phosphate, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, are considerably greater than the actual amount of glucose metabolized through the hexose monophosphate shunt[3].

To determine some of the factors controlling the rate of carbohydrate utilization, the steady state concentrations of TPN and TPNH were determined in the unfertilized and fertilized eggs of *Arbacia* as well as in the eggs of the surf-clam *Spisula Solidissima*. An increase of 3 to 6 fold in the levels of TPNH subsequent to fertilization has been regularly observed.

MATERIALS

TPN, TPNH, DPN, and DPNH were obtained from the Sigma Chemical Company. Alcohol dehydrogenase from yeast, 2 times crystallized was obtained from the

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Abbreviations: TPN, oxidized triphosphopyridine nucleotide; TPNH, reduced triphospohopyridine nucleotide; DPN, oxidized diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

Sigma Chemical Company. Isocitric dehydrogenase was prepared from an acetone powder of pig heart by a modification of the method of MOYLE AND DIXON[7]. Glutathione reductase was prepared according to CIOTTI AND KAPLAN[6].

METHODS

The experiments were carried out at Woods Hole during the summers of 1958 and 1959. Eggs of *Arbacia punctulata* were obtained and handled by methods previously described[4]. *Spisula* eggs were handled according to the methods of ALLEN[5], although much larger numbers of eggs than he used were required for these experiments. *Arbacia* eggs were assayed during 3 stages of development at 20–25°, unfertilized, 1 and 3 h after fertilization and 24 h later when the eggs had reached the pluteus stage. *Spisula* eggs were assayed before and 1 to 3 h after fertilization at 20–25°.

At each stage of development extracts for assay were prepared as follows:

Oxidized pyridine nucleotides

After determination of the volume of eggs by centrifugation, a suspension in filtered sea water was made. Of this suspension 2.0 ml was homogenized for 1 min with 0.2 ml of 50% trichloracetic acid, using a motor driven teflon or glass homogenizer. Following centrifugation, 1.0 ml of the supernatant was added to 1.0 ml of phosphate buffer at 0.1 *M*, pH 7.4 and was brought to pH 7.4 with NaOH. This solution was assayed for TPN according to CIOTTI AND KAPLAN by measuring the decrease in fluorescence induced by methyl-ethyl-ketone with and without TPN-specific isocitric dehydrogenase[6]. TPN was also assayed by a modification of the method of LOWRY, measuring the increase in the native fluorescence of TPNH in the presence and absence of TPN isocitric dehydrogenase[8]. DPN was assayed by the methyl-ethyl-ketone procedure on the same acid extracts, using yeast alcohol dehydrogenase, Tris buffer, pH 10.1, and ethanol 0.5 *M*. In the assay of the oxidized and reduced pyridine nucleotides the Farrand Model A Photofluorometer was used with Corning No. 5860 as primary filter and combining Corning Nos. 4308, 5562 and 3387 as secondary filters. Concentrations of TPN and DPN standards used in the fluorometric assays were previously determined by absorption at 340 mμ after reduction with isocitrate and isocitric dehydrogenase and ethanol and alcohol dehydrogenase respectively.

Reduced pyridine nucleotides

Of a suspension in filtered sea water of a known volume of eggs, 2.0 ml was added to an homogenizing tube containing 25 mg of Na_2CO_3 and mixed by shaking. The tube was heated in a water bath at 100° for 30 sec, homogenized mechanically for 60 sec, and returned to the 100° bath for an additional 60 sec. The homogenate was chilled on ice and centrifuged at 4°. To 1.0 ml of the supernatant was added 1.0 ml of phosphate buffer 0.1 *M* pH 7.4 and the solution cautiously brought to pH 7.4 by the stepwise addition of 0.4 *N* HCl. TPNH was assayed on this solution by the methyl-ethyl-ketone procedure by measuring the increase in fluorescence in the presence of oxidized glutathione and glutathione reductase partially purified from peas[6]. By controlling the concentration of the enzyme, temperature and time of incubation there was no oxidation of DPNH in this assay. DPNH was assayed using methyl-ethyl-ketone by measuring the increase in fluorescence in the presence of $8.3 \cdot 10^{-3}$ *M* acetaldehyde and yeast alcohol dehydrogenase at pH 7.4. The concentrations of TPNH and DPNH standard used in the fluorometric assay were determined by measuring the decrease in optical density at 340 mμ with oxidized glutathione and glutathione reductase and acetaldehyde and alcohol dehydrogenase respectively.

In all assays analyses were performed in duplicate.

RESULTS

In both *Spisula* and *Arbacia* eggs, whether fertilized or unfertilized, the level of TPN was low. In *Spisula* concentrations were 11 and 5 mμmoles/ml of packed cells in unfertilized and fertilized eggs respectively; in *Arbacia* levels were 11 and 14 mμmoles/ml of packed cells in unfertilized and fertilized eggs, respectively. In all instances these values were at the lower limit of sensitivity of the method used. Recovery of TPN added at the instant of homogenization was 100 %.

The values for TPNH levels are summarized in Table I. In *Spisula* eggs recovery of TPNH added at the instant of homogenization was 95–100 %. In *Arbacia* eggs recovery of TPNH was low, from 33–56 %, and the use of an internal standard was required. A known amount of TPNH was added to a duplicate suspension of eggs and the values for *Arbacia* were corrected for the recovery of the added TPNH. In *Arbacia* TPNH levels increased from a mean of 19 mμmoles/ml of cells in the unfertilized eggs to 59 mμmoles in eggs 3 h after fertilization. In each instance in which the fertilized eggs of one batch were compared to the unfertilized cells of the same batch the increase in TPNH was 3 to 7 fold. In Expt. 2 with *Spisula* eggs (Table I) the level of TPNH in the fertilized eggs was low compared to the other experiments. On this day, however, for undetermined reasons, only about 15 % of the eggs underwent cleavage and lost their nuclei despite the appearance of fertilization membranes in over 90 %. In the other experiments with *Spisula* eggs a complete assay of pyridine nucleotide was performed. These values are given in Table II. The relative proportion of TPNH increased dramatically after fertilization from 5 to 30 % of the total pyridine

TABLE I

TPNH CONCENTRATION IN *Spisula* AND *Arbacia* EGGS, EXPRESSED IN MμMOLES/ML PACKED CELLS

Expt.	*Species*	*Minutes after fertilization*			*1440*
		Zero	*60–80*	*150–170*	
1	*Spisula*	23	—	71	—
2	*Spisula*	9	—	12*	—
3	*Spisula*	6	19	41	—
4	*Spisula*	9	—	65	—
1	*Arbacia*	13	—	—	90
2	*Arbacia*	—	—	—	60
3	*Arbacia*	7	57	—	57
4	*Arbacia*	22	61	—	—
5	*Arbacia*	32	—	—	61

* Only 15 % of the cells had undergone cleavage.

nucleotides. Of interest is the low level of DPNH and the slight fall in the DPN concentration consistent with the previous observations of JANDORF AND KRAHL in *Arbacia*[9].

TABLE II

TOTAL PYRIDINE NUCLEOTIDE OF *Spisula* EGGS, EXPRESSED IN $m\mu$MOLES /ML PACKED CELLS

Nucleotide	*Minutes after fertilization*	
	Zero	*150*
DPN	163	140
DPNH	5	11
TPN	11	5
TPNH	9	65

DISCUSSION

The results of these experiments are consistent with the observation that the predominant pathway of glucose utilization in fertilized and unfertilized marine eggs is via the hexose monophosphate shunt. Although the glycolytic pathway becomes more important as development proceeds the absolute amount of glucose metabolized via the hexosemonophosphate shunt increases strikingly after fertilization occurs. The increase in total triphosphopyridine nucleotide (TPN+TPNH) indicates net synthesis from DPN, not merely increase in TPNH at the expense of TPN. Preliminary experiments have demonstrated the presence of DPN kinase in homogenates of fertilized and unfertilized *Arbacia* eggs using a fluorescence assay[10]. The activity of this enzyme, difficult to assay in crude extracts because of the presence of inhibitors[11], was about the same in either stage of development.

It is of interest that the pattern of pyridine nucleotide levels observed in the present studies in fertilized marine eggs is similar to that reported by GLOCK AND McLEAN in the guinea pig ovary[12]. In the mammary gland, another tissue in which the hexose monophosphate shunt is highly active, the steady-state concentrations of TPNH also increases out of proportion to DPNH as lactation proceeds[13]. In the latter tissue, however, levels of oxidized DPN also increase parallel to those of TPNH.

The observed increases in steady-state concentrations of TPNH in a rapidly dividing cell such as the fertilized egg are consistent with current concepts concerning the role of this pyridine nucleotide in synthetic processes[14]. Investigation into the mechanisms relating the changes in pyridine nucleotide levels to the increase in activity of the hexose monophosphate shunt is in progress.

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ON THE FORMATION OF THE TPN REQUIRING GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE DURING THE PRODUCTION OF CHLOROPLASTS IN *EUGLENA GRACILIS*

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SUMMARY

Green Euglena cells possess a TPN-requiring glyceraldehyde phosphate dehydrogenase, in addition to the DPN-requiring enzyme. The dark-grown, colorless cells contain only the DPN enzyme. In resting colorless cells exposed to light, the course of formation of the TPN enzyme follows closely that of chlorophyll. Removal of light during this process results in a cessation of both enzyme production and chlorophyll synthesis. Cultivation of the green cells at 34° in the light produces an inhibition of chloroplast synthesis, and return of the cells to a lower temperature brings about a rapid resumption of chloroplast synthesis after a lag period of one to two days. The production of the TPN enzyme follows very closely that of the chloroplasts, both during the inhibition and the recovery. The synthesis of the DPN enzyme, on the other hand, is linked to the multiplication of the cells during this process.

INTRODUCTION

Green plant tissues have been found to contain a GPDase that requires TPN[1,2]. This enzyme is absent from the non-photosynthetic plant tissues, which contain the DPN dependent enzyme. The TPN activity has been shown further to be the result of two different enzymes; one which requires inorganic phosphate or arsenate, and another one that does not have these additional requirements[3,4]. HAGEMAN AND ARNON have studied the formation of the TPN enzymes during the germination of pea seeds[5]. The appearance of the TPN dependent activity in the shoot was found to occur only in the presence of light. This process thus appears to be analogous to the induced enzyme formation in bacteria.

In conjunction with studies in this laboratory of the chemical events accompanying the formation of the photosynthetic apparatus in dark-grown (colorless) *Euglena gracilis* cells exposed to light[6,7], it appeared of interest to follow the emergence of a specific enzyme connected with photosynthesis. The TPN linked GPDase has

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Abbreviations: GPDase, glyceraldehyde-3-phosphate dehydrogenase; TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; FDP, fructose-1,6-diphosphate; G6P, glucose-6-phosphate.

been reported to be present in the green *Euglena gracilis* cells and absent from the colorless and the permanently bleached organisms[8]. We have studied the production of this enzymic activity during the formation of chloroplasts in *Euglena*. The use of a unicellular photosynthetic organism has enabled us to study the formation of this enzyme under strictly controlled conditions, and has revealed a very tight regulation of enzyme production during the synthesis of the chloroplasts.

EXPERIMENTAL

Materials

Fructose-1,6-diphosphate, Mg salt, and cysteine hydrochloride were purchased from Schwarz Laboratories, Mount Vernon, N.Y.; DPN, TPN, and glucose-6-phosphate, Na salt, from Sigma Chemical Co., St. Louis; DL-glyceraldehyde-1-bromide-3-phosphoric acid, dioxane complex, from California Corporation for Biochemical Research, Los Angeles; crystalline aldolase from Nutritional Biochemical Corporation, Cleveland.

Cultures: Euglena gracilis, strain z was used. The broth and "resting" media have been described previously[6].

Preparation of extracts

The cell samples were collected by centrifugation, washed with water and stored in a freezer. The following extraction procedure, adapted from that used by HAGEMAN AND ARNON[5], yielded reproducible and presumably quantitative amounts of enzyme from the cells. All the operations were carried out in the cold room. Acetone was cooled to the temperature of a dry ice–ethanol bath, then added to the samples. The frozen cell mass was disrupted by vigorous stirring until a homogeneous suspension in the acetone was obtained. The material was then collected on a small Buchner funnel and suction was continued for a few minutes in order to eliminate the residual acetone. The resulting dry powder was transferred into a centrifuge tube. At this stage, the material could be stored for a few days in the freezer. The active extracts were obtained by suspending the acetone-treated samples in a 0.02 *M* NaCl–0.0015 *M* ethylenediaminetetraacetate, pH 6.4, solution, stirring the suspension for 1 min, then centrifuging. The GPDase activities in the supernatant were rather unstable, and the assays had to be done within 15 min after the final extraction. It was found later that addition of neutralized cysteine hydrochloride to the extracts improved the stability of the enzymes.

Enzyme assays

FDP in conjunction with aldolase was used as the substrate for the GPDase measurements[9]. The assay mixture consisted of 0.07 *M* tris (hydroxymethyl) aminomethane, pH 8.5, 0.007 *M* cysteine hydrochloride, 0.007 *M* Na arsenate, 60 μg/ml of aldolase, 70 μg/ml of TPN, 0.008 *M* FDP, and extract in a total volume of 3 ml. DPN was substituted for TPN when the DPN requiring enzyme was determined, and FDP was replaced by G6P in the assay for G6P dehydrogenase. The assay mixture minus FDP was incubated with the extract for about 5 min; a blank without extract was also incubated. The reaction was started by adding FDP to both the test mixture and the blank, and 1 min later the increases in O.D. at 340 mμ were measured every

30 sec for 2 min. The activity was expressed as the increase in O.D. units/min. The rate of increase in O.D. was usually constant for about 5 min in the case of the TPN-requiring enzyme. With the DPN enzyme, however, this rate decreased rapidly, and the measurements were recorded for only 1.5 min. The activity was fairly proportional to the amount of enzyme in the range of 0.01 to 0.05 O.D. units, and the amounts of extracts used for the assay were adjusted to give activities within this range. The test as used does not distinguish between the two different TPN requiring GPDases, and the results obtained therefore represent the overall TPN-GPDase activity.

Cell fractionation

One volume of 10 % sucrose in 0.01 *M* Tris buffer pH 7.6 and 0.005 *M* $MgCl_2$ was added to an equal volume of packed cells, freshly collected. The cell suspension was treated in the cold for 3 min in a glass tissue grinder equipped with a tight fitting teflon pestle. Six volumes of the sucrose solution were then added to the disrupted cells and the suspension was centrifuged at 1000 × *g* for 20 min. The sediment, containing both chloroplasts and unbroken cells, was resuspended in fresh sucrose solution and the cells were removed by repeated centrifugations at 140 × *g* for 2 min. The chloroplasts were then resedimented at 1000 × *g* for 20 min. The supernatant from this last centrifugation ("chloroplast-supernatant") was saved. The combined mitochondria and microsomes were obtained by centrifugation of the first supernatant at 100,000 × *g* for 60 min. The resulting supernatant was used as "soluble fraction". All fractions were stored in the freezer. This fractionation procedure yielded apparently intact chloroplasts. Some plastid breakage occurred,as suggested by the presence of chlorophyll in the 1000 × *g* supernatant.

For the enzyme assays, the particulate fractions were either treated with cold acetone before extraction, or extracted without any acetone treatment. The latter procedure gave somewhat higher enzyme activities.

Analytical methods

For protein measurements, cell samples were successively extracted with cold 5 % trichloroacetic acid and with ether–alcohol (3:1), dissolved in 0.1 *N* NaOH and determined by a biuret method[10]. Cell counts were done in a hemocytometer after immobilization with a little chloroform. Chlorophyll was determined by O.D. measurements at 665 mμ of ethanol extracts[6].

RESULTS

Enzymic activities in green and colorless cells

The use of FDP as substrate with crude extracts of plant tissues could lead to the formation of G6P and subsequent reduction of TPN with the help of G6P dehydrogenase[11]. The fact that G6P dehydrogenase does not require sulfhydryl groups makes the estimation of this side reaction possible. The results in Table I indicate that the extracts from green cells exhibit very little activity with FDP as the substrate in the absence of cysteine. The reduction of TPN by G6P, on the other hand, is completely independent of cysteine. The small activity obtained with FDP and TPN in the absence of cysteine could therefore be a measure of the contaminating G6P dehydro-

TABLE I

GLYCERALDEHYDE-3-PHOSPHATE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITIES OF GREEN AND COLORLESS *Euglena* CELLS

Activities expressed as O.D. units/mg of total cell protein.

Type of cells	*Substrate*	*Cofactor*	*Activity*	
			No cysteine	*Cysteine added*
Green	FDP	TPN	0.011	0.083
	FDP	DPN	0.005	0.43
	G6P	TPN	0.026	0.025
Colorless	FDP	TPN	0.012	0.015
	FDP	DPN		0.53
	G6P	TPN		0.015

genase activity, and the assay method used in this investigation appears sufficiently specific for the measurement of the TPN requiring GPDase activity. As a verification, FDP and glyceraldehyde-3-phosphate were compared as substrates, and similar values for the rates of reduction of TPN were obtained*. In the colorless cells, the TPN activity is essentially independent of cysteine, and is equal to the cysteine independent activity of the green cells. This most likely does not represent GPDase activity.

The results of Table I indicate that the amount of DPN dependent GPDase activity of the green cells is 5 to 6 times higher than the corresponding TPN activity. The DPN activity of the colorless cells appears slightly higher than that of the green cells.

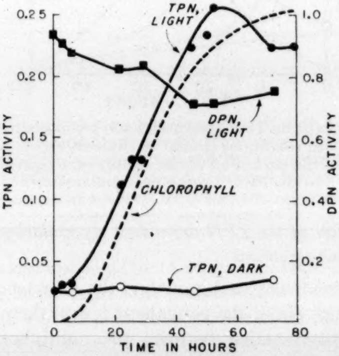

Fig. 1. Glyceraldehyde-3-phosphate dehydrogenase activities during the formation of chloroplasts in resting colorless cells. Cells from 2-l culture grown in the dark collected, suspended in 400 ml resting medium. One-half of suspension exposed to light, (full circles); other portion kept in the dark (open circles). 20-ml samples collected for the enzyme assays. Chlorophyll (broken line) determined on separate samples. TPN activity: circles; DPN activity: squares.

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* HAGEMAN AND ARNON, in their study of the GPDase activity in germinating pea seeds, also concluded that FDP was a satisfactory substrate for their extracts[5].

Appearance of TPN glyceraldehyde-3-phosphate dehydrogenase during the formation of chloroplasts in colorless cells

The course of formation of the TPN GPDase activity in resting colorless cells exposed to light is described in Fig. 1. The formation of the TPN enzyme follows closely the production of chlorophyll in the cells. The small drop in activity near the completion of the greening process was encountered in most experiments. The DPN activity decreases somewhat during the process.

Removal of the light source before the completion of the greening process results in an immediate cessation of chlorophyll synthesis[12]. The production of the TPN GPDase activity is also blocked when the cells are returned to the dark (Fig. 2).

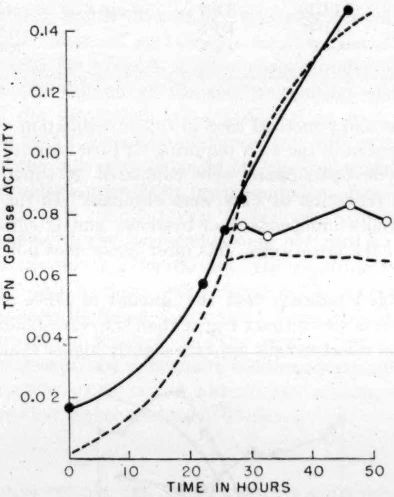

Fig. 2. Effect of removal of light on TPN glyceraldehyde-3-phosphate dehydrogenase production in colorless cells. Experimental conditions similar to those in Fig. 1. After 26 h in the light, part of the suspension returned to the dark. Full circles: cells in the light; open circles: cells returned to the dark; broken line: chlorophyll.

Regulation of the formation of the TPN-dependent glyceraldehyde-3-phosphate dehydrogenase during the bleaching treatment

It has been shown previously that growth of the *Euglena* cells at 34.5° (bleaching temperature), which brings about the permanent loss of their ability to form chloroplasts, causes a disproportionation between the multiplication of cells and the production of plastids[12]. This results in organisms containing progressively fewer chloroplasts. We have followed the formation of both the TPN and the DPN enzymes during this process. Under non-growing conditions, the bleaching temperature causes only a slight retardation in the development of chloroplasts in colorless cells. Similarly its effect on the formation of the TPN enzyme is very small (see Table II). In Fig. 3, the course of events during growth at the bleaching temperature is described. The rise in the TPN enzyme follows very closely the formation of chlorophyll. When the

TABLE II

EFFECT OF THE BLEACHING TEMPERATURE OF THE FORMATION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE IN RESTING COLORLESS CELLS

Cells from 1 l of dark grown culture collected, washed, suspended in 300 ml resting medium. One portion kept at 34.5°, the other one at room temperature. Both suspensions kept in the light.

Time (h)	*Chlorophyll*		*TPN GPDase activity*	
	Room temperature	*Bleaching temperature*	*Room temperature*	*Bleaching temperature*
0	0.007	0.007	0.03	0.03
21	0.50	0.37	0.14	0.12
47	0.74	0.75	0.19	0.22

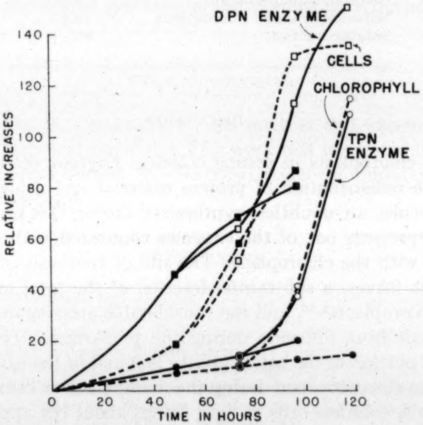

Fig. 3. Formation of the glyceraldehyde-3-phosphate dehydrogenases during the bleaching treatment. 6 ml green culture inoculated into 600 ml broth, kept in the light at 34.5°. After 49 h, portion transferred aseptically to another flask and kept at room temperature. Full circles and squares: culture at 34.5°; open circles and squares: portion placed at room temp.; full lines and circles: TPN enzyme; full lines and squares: DPN enzyme; broken lines and circles: chlorophyll; broken lines and squares: cells.

cells are returned to room temperature, a rapid formation of chloroplasts takes place after a lag period of 1 day[12]. Again the production of the TPN enzyme parallels the increase in chlorophyll. The DPN enzyme, on the other hand, behaves like a normal cellular constituent, as evidenced by a comparison of the curves for cellular multiplication and DPN enzyme production.

Distribution of the TPN GPDase in the cell fractions

Most of the TPN GPDase activity was found in the soluble fraction (see Table III). The possibility that the enzyme was originally present in the chloroplasts, and had leaked out during the fractionation procedure, must, however, be considered. The disruption of the cells lasted 3 min and the subsequent centrifugation of the chloroplasts 20 min. The enzyme molecules could have diffused from the plastids into the soluble fraction during this 25-min period. Any subsequent enzyme diffusion should have led to activity in the "chloroplast-supernatant" fraction. The lack of activity in this fraction indicates that if any diffusion had occurred, it was completed within the initial 25-min period. Although the results tend to indicate that the enzyme is normally present in the soluble fraction of the cytoplasm, a loose association with the chloroplasts is also possible.

TABLE III

DISTRIBUTION OF TPN GPDASE IN CELL FRACTIONS OF *Euglena*

Fraction	*% of total activity*
Chloroplasts	9
Chloroplast-supernatant	2.5
Mitochondria and microsomes	1.5
Soluble fraction	87

DISCUSSION

The formation of chloroplasts in resting colorless *Euglena* cells exposed to light is accompanied by a redistribution of protein material and a high protein turnover. New protein molecules are doubtless synthesized during this process, and the TPN-linked GPDase represents one of the enzymes connected with photosynthesis that are formed along with the chloroplasts. The site of synthesis of this enzyme is not known. In spinach leaves, a substantial fraction of the total enzymic activity can be found in the chloroplasts[4,13], and the considerable activity in the soluble fraction is thought to result from diffusion during the preparation. In the green *Euglena* cells, only a small portion of the total activity is found in the isolated plastids, but a rapid diffusion into the cytoplasm during the manipulations cannot be excluded.

Exposure of the colorless cells to light brings about the appearance of the TPN enzyme, and removal of the light stimulus results in the cessation of enzyme formation. This behavior is similar to that of many induced enzymes in bacteria. In this case, however, induction by light initiates a complex chain of events, and it is doubtful that light is the primary inducer of the TPN-linked GPDase. Since one of the effects of light during photosynthesis is the production of reduced TPN[14], it is tempting to speculate that this substance acts as an inducer for the formation of the TPN enzyme. This would require that functional photosynthesic units are formed soon after exposure of the colorless cells to light, since the production of the enzyme begins very early in the greening process.

The cessation of enzyme production when light is removed can be explained reasonably by assuming that photosynthetic activity is necessary for its synthesis. In the case of the bleaching treatment, however, chloroplast formation is inhibited in the presence of light. Photosynthesis presumably occurs under these conditions, and the bleaching temperature itself has very little effect on the production of the enzyme (Table II). Therefore one should not necessarily expect a strict relation between amount of enzyme formed and number of chloroplasts produced during the bleaching treatment. Yet the increases in TPN enzyme and chloroplasts appear strictly linked under these conditions. Also after return of the culture to room temperature, both processes show the same lag period followed by an identical rapid rise.

The close relation between the production of TPN-linked GPDase and the formation of chloroplasts during partial bleaching followed by recovery suggests that it is the quantity of plastid structure, rather than the activity of the plastids, that determines the extent of enzyme synthesis. An understanding of the manner in which such a regulation could operate must await more definite evidence on the localisation of the enzyme in the cells, and possibly some knowledge of the site of TPN–GPDase synthesis. If the enzyme were produced in the plastids and remained associated with their structure, a direct control of the amount of enzyme in each plastid could be visualized. The problem of regulation should, however, be much more intricate if the localization and the site of synthesis of the enzyme were outside the chloroplast structure.

ACKNOWLEDGEMENTS

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SOME HAEM PROTEIN-LINKED PYRIDINE NUCLEOTIDE OXIDATION SYSTEMS IN *RHODOSPIRILLUM RUBRUM**

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SUMMARY

Ammonium sulfate fractionation of a crude citrate extract obtained from lyophilized cells of *R. rubrum*, a facultative photoheterotrophe, yields a soluble enzyme preparation which contains "bound" bacterial cytochrome c ("cytochrome c_2") and variant heme protein ("RHP"). The following light-insensitive reactions, enzymically-catalyzed by this preparation, have been noted: (a) TPNH oxidase (b) DPNH oxidase (c) TPNH-cytochrome c_2 reductase (d) DPNH-cytochrome c_2 reductase (e) TPNH-RHP reductase (f) DPNH-RHP reductase (g) TPNH-2,6-DCPI reductase and (h) DPNH-2,6-DCPI reductase. The reaction between DPNH and 2,6-DCPI indicates 10-fold purification of the enzyme with respect to the initial crude extract. Using this preparation, some characteristics of these catalyzed reactions have been determined.

The evidence obtained from studies of heat inhibition and variations with pH suggests that reactions (g) and (h) are catalyzed by different enzymes, the latter being much more reactive. The rates of oxidase reactions (a) and (b) are very small, being 1/300 and 1/50 respectively of those found for DPNH and TPNH using 2,6-DCPI as H-acceptor.

The bound RHP is rapidly oxidized, whereas cytochrome c_2 is only partially oxidized when the enzyme preparation is aerated. When DPNH is added in air, only cytochrome c_2 is reduced. Under anaerobic conditions, both endogenous heme proteins are reduced to the maximum extent, but at different rates. These preliminary studies are in accord with an oxidation scheme in which dehydrogenation of the substrate DPNH proceeds via RHP to both cytochrome c_2 and oxygen as hydrogen acceptors.

INTRODUCTION

The participation of haem proteins in photosynthesis is well authenticated[1,2]. Studies relevant to an understanding of mechanisms involved have been made, particularly a number of investigations on the metabolism of purple photosynthetic bacteria,

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Abbreviations: DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; 2,6-DCPI, 2,6-dichlorophenol-indophenol; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide.

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such as the facultative photoheterotrophe, *Rhodospirillum rubrum*, and the photoanaerobe, *Chromatium*. These have included exploratory tests for the occurrence in cell-free extracts of light-sensitive and light-insensitive oxidase activities associated with the bacterial haem proteins[3–5], adaptive synthesis of haem proteins during illumination of cell suspensions[6], simultaneous light-stimulated oxido-reductions[7,8], photophosphorylation[9–13], and *in vivo* studies of light-induced shifts in the oxidation states of cellular haem proteins[14–16]. However, data bearing on partial reactions at the enzyme level are meager, and the enzymic basis for the action of haem proteins remains obscure.

In *Rhodospirillum rubrum*, most of the haem proteins present in the photoactive portions of the cell can be accounted for as a mixture of two compounds[4]. One of these is a *c*-type cytochrome ("cytochrome c_2") and the other is a variant haem protein, called "RHP". Both of these proteins have been isolated and procedures established for their preparation in relatively large amounts with high purity[17,18] and even in crystalline form[18]. The availability of these haem protein preparations together with advances in technology of enzyme isolation procedures makes feasible a re-investigation and extension of earlier work which was done using very crude preparations of pyridine nucleotide-oxidases[3].

In this paper we will present results of orientation experiments on partially purified enzyme systems which confirm previous suggestions[3], based on the early work with the cruder preparations, that DPNH- and TPNH-linked-RHP reductases exist which are coupled to reduction of cytochrome c_2.

MATERIALS AND METHODS

R. rubrum was grown anaerobically in 20-l bottles under conditions of continuous illumination at room temperature (approx. 30°) in the synthetic medium described by COHEN-BAZIRE, SISTROM AND STANIER[19]. After incubation for 3–4 days, the cells were harvested using a Sharples centrifuge, washed with water, lyophilized and stored at —5°.

Crystalline cytochrome *c* was prepared according to the method of NOZAKI *et al.*[20], using as starting material a horse heart muscle cytochrome *c* sample commercially available from Sigma Chemical Company, St. Louis, Mo.

To obtain a crude enzyme preparation, the lyophilized powder of light-grown cells was mixed in a Waring blendor with 0.1 *M* sodium citrate in the proportion 10 ml per gram of cells. The mixture was adjusted to pH 7 with 20 % aqueous KOH, then extracted with continuous stirring in a cold room (4–5°) for one day. To the extract, solid ammonium sulfate was added to 40 % saturation, followed by centrifugation in the cold at 18,000 rev./min for 2 h in a Servall automatic superspeed centrifuge. To the supernatant, solid ammonium sulfate was further added up to 65 % saturation. The resulting precipitate was collected by centrifugation and resuspended in 10 volumes 45 %-saturated aqueous ammonium sulfate. The suspension was centrifuged at 40,000 rev./min for 2 h in a Spinco preparative centrifuge. The resulting supernatant was supplemented with saturated aqueous ammonium sulfate to 65 % saturation. After centrifuging, the precipitate was dissolved in distilled water, then dialyzed against distilled water in a cold room for two days with several changes of outside water. The dialyzed solution (I) was lyophilized (II).

Approximately 50 mg of (II) was obtained from 10 g of the lyophilized cells used as the starting material. The lyophilized powder was stored at —5°. Just before use, distilled water was added to the lyophilized powder to make a solution containing approximately 50 mg/ml. The mixture was centrifuged at 40,000 rev./min for 2 h using a Spinco preparative centrifuge. A large amount of colorless material was precipitated by this centrifugation, and was easily removed by this procedure. The resulting clear, reddish brown supernatant was used as an enzyme preparation (III). The purification steps are summarized in Table I.

TABLE I

SUMMARY OF PURIFICATION OF COENZYME – 2,6-DCPI REDUCTASE FROM *R. rubrum*

Purification steps	*Volume (ml)*	*Total extinction**		*Protein (mg)*	*Total enzyme units* $(-\Delta E^{DPNH}_{600\,m\mu}/min)$	*Specific activity (Enzyme units/mg protein)*
		$E_{275\,m\mu}$	$E_{256-259\,m\mu}$			
Lyophilized cells	—	—	—	50,000**	—	—
Extract	454	97,000	160,000	4,090	139	0.035
45–65 % saturated ammonium sulfate (I) fraction	54	1,260	1,820	221	94	0.42
Lyophilized enzyme (II)	—	—	—	241**	87	0.36
Enzyme preparation (III)	5.4	945	1,260	151	70	0.46

* Absorption maximum at 256–259 mμ.
** Dry weight.

Absorbancies were measured at approx. 22°, using a Cary ratio-recording spectrophotometer, model 14, furnished with a potentiometer providing two absorbancy scales-0.0–1.0 and 1.0–2.0. Anaerobic spectrophotometric measurements were made with a Thunberg type cuvette. In a typical experiment, the cuvette (1-cm optical path) was first evacuated, then flushed with helium gas. This procedure was repeated until no more bubbling occurred despite vigorous shaking of the cuvette during final evacuation.

Percentage saturation of ammonium sulfate was calculated according to the table given by GREEN AND HUGHES[21] when solid salt was used to make a solution of appropriate concentration. When saturated aqueous ammonium sulfate was added, an amount of salt solution saturated at room temperature (approx. 22°) was added as calculated from simple dilution. All salting-out procedures were carried out at pH 6.5–7.0.

Protein concentration was determined by a modified biuret reaction[22] in which the influence of salt was counteracted by the use of the same concentration of the salt solution in controls.

To measure coenzyme-2,6-DCPI reductase activity, the following reaction mixture was used: an appropriate amount of enzyme preparation (see Fig. 1); 0.5 ml of 0.1 *M* buffer tris(hydroxymethyl)aminomethane-HCl, "Tris", pH 8.0, used in all assays unless indicated otherwise; 0.1 ml of 10^{-3} *M* aqueous 2,6-DCPI; 0.1 ml of 0.006 *M* reduced coenzyme (TPNH or DPNH); and water to make the total volume 1.2 ml. The reaction was started by rapid addition of the reduced coenzyme solution, and decrease in absorbancy at 600 mμ was followed at 22° in the air. Automatic recording was started precisely 10 sec after the beginning of the reaction. Readings were made for the following 30 sec.

The enzyme activity, $-\Delta E^{\mathrm{DPNH}}_{340\,\mathrm{m}\mu}$/min, was expressed simply as three times the experimental readings from 20 sec to 40 sec after the beginning of reaction. One enzyme unit was defined as the amount of enzyme giving a value of 1.0 in $-\Delta E^{\mathrm{DPNH}}_{600\,\mathrm{m}\mu}$/min. Specific activity of the enzyme was defined as units/mg of protein.

To measure reduced coenzyme oxidase activity, the same experimental conditions as for the reduced coenzyme–2,6-DCPI reductase activity were used, except that water was added instead of the dye. The oxidation of reduced coenzyme, $-\Delta E^{\mathrm{DPNH}}_{340\,\mathrm{m}\mu}$/min, was assayed by change in absorbancy at 340 mμ.

The oxidation of the ascorbate-reduced horse-heart cytochrome *c* was measured according to the method of KAMEN AND VERNON[3], except that the enzyme preparation was used instead of the sonic extract.

RESULTS

Oxidation of reduced coenzymes by Preparation (III)

Both DPNH and TPNH could be oxidized by 2,6-DCPI in the presence of (III). The rate of oxidation of DPNH was an order of magnitude higher than that of TPNH, as shown in Fig. 1. The relative rates varied somewhat in different batches of (III). In the range of the enzyme concentrations used, the activities showed linear dependence with amount of the enzyme preparation used. All of the following experiments on reduced coenzyme–dye reductase activity were confined to this range of enzyme concentration.

If 2,6-DCPI was omitted from the reaction mixture, oxidation of both coenzymes could still proceed. The rates for DPNH and TPNH oxidation were roughly one-three hundredth and one-fiftieth that in the presence of the dye. The activities of the enzyme preparation for the reduced coenzyme–dye reductase and the reduced coenzyme oxidase were insensitive to irradiation with light of wave lengths effective in bacterial photosynthesis.

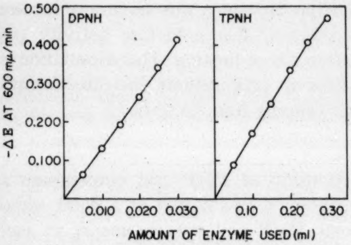


Fig. 1. Reduced coenzyme – 2,6-DCPI reductase activities of enzyme preparation III.

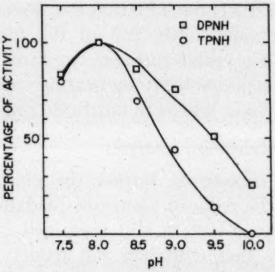


Fig. 2. Influence of pH on the reduced coenzyme – 2,6-DCPI reductase activity of enzyme preparation III. Tris buffer (0.1 *M*) was used for the pH range from 7.5 to 9.0, and glycine–NaOH buffer (0.1 *M*) for the higher pH range.

The optimal pH of the reduced coenzyme–2,6-DCPI reductase activity was found to be 8 for both DPNH and TPNH, as shown in Fig. 2.

The marked difference in behavior of the two curves in the alkaline pH-range shown in Fig. 2, could indicate that two different enzymes were involved. This conclusion was consistent with the fact that the ratio of the two reductase activities was altered during the purification steps given in Table I.

Effect of heating on oxidation of reduced coenzymes by (III)

When (III) was heated at 50° and at pH 8 (0.08 M Tris buffer), the reduced coenzyme–2,6-DCPI reductase activity was first markedly stimulated, then gradually depressed, as shown in Fig. 3. After 20 min of heating, the enzyme preparation oxidized neither DPNH nor TPNH in the absence of the dye. However, this amount of heating gave maximal activation of the reduced coenzyme–dye reductase activity (see Fig. 3). No visible turbidity appeared even after 1 h of heating. The stimulation of dye reductase activity by heating varied in different preparations, increases in ratio of activity of heated to untreated preparations ranging from 50 to 90%.

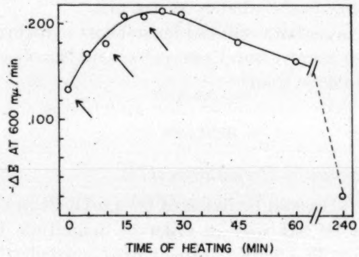


Fig. 3. Effect of heating at 50° on DPNH – 2,6-DCPI reductase activity of enzyme preparation III. One volume of III was mixed with five volumes of 0.1 M Tris buffer (pH 8.0), then the mixture was heated at 50°. From the mixture, aliquots were pipetted at times indicated and immediately chilled in a slurry of ice–water. The reduced coenzyme – 2,6-DCPI reductase activity was not changed when the preparation was maintained in the chilled state for one day. Hence DPNH – 2,6-DCPI reductase activity of stored aliquots could be assayed at the same time after conclusion of the 4-h heating experiment. Arrows refer to aliquots withdrawn for assay of DPNH-cytochrome c_2 reductase (see Fig. 7).

Spectrophotometric analyses

To investigate further the mutual relationship of RHP and cytochrome c_2 during the reduced coenzyme oxidations, purification was halted at a point where the enzyme system still contained endogenous RHP and cytochrome c_2 in such amounts that oxidation and reduction of these haem proteins could be spectrophotometrically assayed readily. The neutral pH indicated in the preparative procedure (Table I) was dictated by the need to avoid excessive denaturation of the oxidase system which was found to become inactivated rapidly at pH <7. This enzyme preparation (III) contained less cytochrome c_2 than RHP. Thus, it was calculated that $4.6 \cdot 10^{-4}$ M RHP and $3.5 \cdot 10^{-5}$ M cytochrome c_2 were present, based on preliminary values of molecular extinction of RHP obtained by BARTSCH AND KAMEN[17] together with a value for the molecular extinction of the reduced cytochrome c_2, at 550 mμ, of $28 \cdot 10^6$. This result followed from the fact that RHP was more readily precipitated than cytochrome c_2, at the concentrations of ammonium sulfate used (Table II).

Before the lyophilization of the enzyme preparation (See METHODS), more than 80% of the cytochrome c_2 present in the enzyme preparation existed in its reduced form. The lyophilization changed approximately 60–70% of the cytochrome c_2 into its oxidized form. On the other hand, the RHP present in the enzyme preparation was always found in its oxidized form at the first step in which RHP could be spectrophotometrically detected.

When DPNH was added to (III) in air, the endogenous cytochrome c_2 was rapidly reduced, while the endogenous RHP remained in its oxidized form. The more DPNH was added to the enzyme preparation, the more reduced the cytochrome c_2 became.

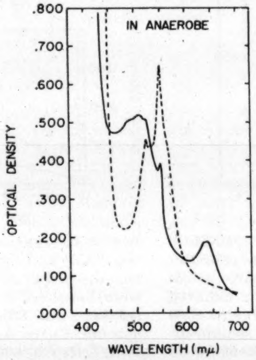


Fig. 4. Anaerobic reduction by DPNH of the RHP and cytochrome c_2 present in enzyme preparation III. A Thunberg type cuvette contained the following components: 0.30 ml of the enzyme preparation, 1.00 ml of Tris buffer (0.1 M, pH 8.0), 0.20 ml of 0.006 M DPNH and water to make the total volume 3.00 ml. DPNH was added from the side arm of the cuvette after the evacuation of the cuvette. ———, absorption spectrum of the enzyme preparation before the addition of DPNH. The reading was carried out from 700 mμ to the lower wavelengths, time required being 10 sec for the spectrophotometer to scan 10 mμ. ------, absorption spectrum of the enzyme preparation reduced by DPNH. The scanning was begun 5 min after the mixing of DPNH from the side arm. No corrections were made for concentration changes caused by addition of DPNH and evacuation of the cuvette.

If DPNH was added to (III) under anaerobic conditions, both the RHP and cytochrome c_2 were reduced to the same extent (see Fig. 4). This degree of reduction was equal to that obtained by addition of sodium dithionite in excess. The reduction of the cytochrome c_2 was much faster under anaerobic conditions, in spite of the negligible auto-oxidizability exhibited by pure cytochrome c_2. Since the normal redox-potentials of RHP and cytochrome c_2 have been reported to be approximately 0.0 and 0.3 V, respectively[4,16], this fact indicated that (III) contained at least one enzyme which could catalyze the reduction of RHP itself, and that the reduction of RHP could not have been effected to any appreciable extent *via* reduced cytochrome c_2.

Kinetics of the reduction of the RHP and cytochrome c_2 present are shown in Figs. 5-A and 5-B. It was assumed that the decrement of O.D. at 640 mμ resulted wholly from disappearance by reduction of the absorption peak at this wavelength of the oxidized RHP (Curve A in Fig. 5-A). Both RHP and cytochrome c_2 contributed to the absorption peak at 550 mμ. Therefore, the increment of O.D. at 550 mμ was assumed to result from the reduction of both endogenous RHP and cytochrome c_2 (Curve A in Fig. 5-B). The comparison in rate between Curves A in Fig. 5-A and Fig. 5-B indicated that the cytochrome c_2 was much more rapidly reduced by DPNH than the RHP.

During this work, cytochrome c_2 and RHP were prepared in their crystalline forms from LG cells of *R. rubrum*[18]. If pure cytochrome c_2 was added to (III) under anaerobic conditions, the reduction of the RHP by DPNH showed a longer latent period than in the case of the enzyme preparation without such addition, as shown by the dotted line in Fig. 5-A. If air was admitted into the cuvette containing the enzyme preparation reduced by DPNH, the RHP was rapidly oxidized. Gentle shaking of the cuvette sufficed to completely oxidize the RHP. On the contrary, only 5–10 % of the cytochrome c_2 was oxidized despite vigorous shaking of the cuvette. It was also noted that reduced horse heart cytochrome *c* could be slowly but steadily oxidized by (III) in air.

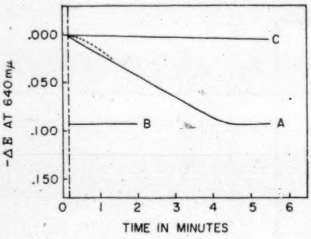


Fig. 5-A. Anaerobic reduction by DPNH of the RHP present in the enzyme preparation. Experimental conditions were the same as for Fig. 4, except that the reaction was followed at a fixed wavelength, 640 mμ. Curve A, with the enzyme preparation; Curve B, sodium dithionite added at the end of experiment traced in Curve A; Curve C, with the enzyme preparation previously heated at 50° for 10 min (see Fig. 3). For explanation of dotted line see text.

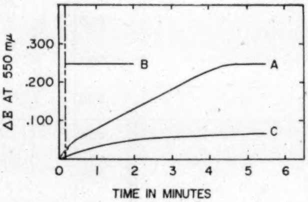


Fig. 5-B. Anaerobic reduction by DPNH of the RHP and cytochrome c_2 present in the enzyme preparation. Experimental conditions were the same as for Fig. 5-A, except that the reaction was followed at a fixed wavelength, 550 mμ. Curve A, with the enzyme preparation; Curve B, sodium dithionite added at the end of experiment traced in Curve A; Curve C, with the enzyme preparation previously heated at 50° for 10 min (see Fig. 3).

When (III) was heated at 50° for 10 min, the endogenous RHP was reduced hardly at all by DPNH under anaerobic conditions, while cytochrome c_2 present was slowly reduced, as shown in Fig. 6. The kinetics of the reductions of both hemoproteins are shown in Curves C of Fig. 5-A and Fig. 5-B. If the initial velocities in increment of absorbance at 550 mμ were compared for (III) and for the heated enzyme preparation, both having been supplemented by addition of the crystalline oxidized cytochrome c_2, then it was found that reduction of the cytochrome c_2 by DPNH was greatly diminished in rate by the heating, as shown in Fig. 7. The same general result was observed using TPNH. Heating of (III) at 50° for 20 min inactivated the aerobic oxidation of reduced coenzymes in the absence of 2,6-DCPI and the anaerobic reduction of the RHP by the reduced coenzymes.

Further purification of coenzyme–2,6-DCPI reducing enzyme

The enzyme could be further purified by repeated fractionation with saturated

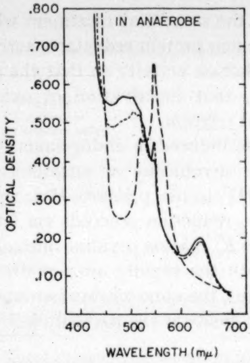


Fig. 6. Anaerobic reduction by DPNH of the cytochrome c_2 and RHP present in the enzyme preparation previously heated at 50° for 10 min. Experimental conditions were the same as for Fig. 4, except that the heated enzyme preparation was used. ———, before the addition of DPNH *in vacuo* from the side arm of the cuvette; -----, after the addition of DPNH (the scanning was started 8 min after the addition of DPNH); – – – – –, sodium dithionite added in excess after termination of scanning.

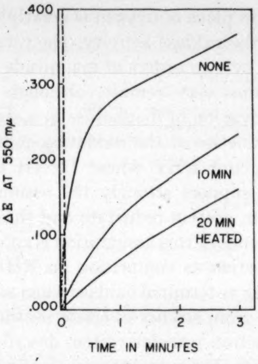


Fig. 7. Anaerobic reduction by DPNH of the cytochrome c_2 present in the enzyme preparation which was externally supplemented with cytochrome c_2. Experimental conditions were the same as for Fig. 5-B, except that $1.30 \cdot 10^{-2}$ μmoles of crystalline cytochrome c_2 in its oxidized form was externally added to the reaction mixture. Curve None, with the enzyme preparation without heating; Curve 10 min, with the enzyme preparation heated at 50° for 10 min; Curve 20 min, with the enzyme preparation heated at 50° for 20 min (see Fig. 3). It was assumed that the initial rates resulted from the reduction of cytochrome c_2 but not RHP (refer text and see the dotted line in Fig. 5 A).

aqueous ammonium sulfate, as shown in Table II. The 55–60% saturated ammonium sulfate fraction showed the strongest reduced coenzyme–2,6-DCPI reductase activity of the four fractions obtained. This fraction contained the least amounts of RHP and cytochrome c_2. The specific enzyme activity was enriched a hundred-fold compared to that of the first extract (see Table I).

The aerobic DPNH-oxidizing activity was lost from all fractions after the ammonium sulfate fraction. However, a mixture of the 55–60% and 78–100% saturated ammonium sulfate fractions showed aerobic DPNH-oxidizing activity approximately 0.2 as much as the original enzyme solution, indicating the possibility that the oxidation might occur *via* endogenous RHP contained in the 78–100% fraction. Critical experiments on this problem will be repeated when much more active preparations are available.

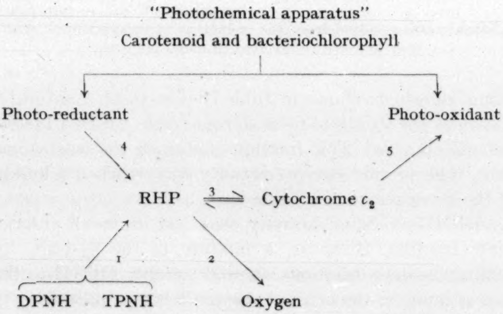
DISCUSSION

The experiments presented in this report extend previous observations relating to the light-insensitive haem protein mediated pyridine nucleotide oxidases[3]. It is seen that the diaphorase (dye reductase) activity can be differentiated from the oxidase activity solely by heat treatment. Thus, short-term heating at 50° abolishes oxidase activity whereas the diaphorase action is markedly stimulated. Further, the ability of the pyridine nucleotide oxidases to use either RHP or cytochrome c_2 as terminal acceptors in place of oxygen is greatly diminished by the same heat treatment which abolishes the oxidase activity. The total oxidase, or haem protein reductase, activity is smaller by two orders of magnitude than the diaphorase activity so that the possibility seems very remote, although not excluded, that inactivation of oxidase causes activation of diaphorase in a strictly reciprocal relation.

The kinetics of the oxidation–reduction sequence induced in endogenous cytochrome c_2 and RHP where DPNH is added either aerobically or anaerobically, appear to support strongly the assumption that RHP is the primary H-acceptor of the haem protein reductase and that cytochrome c_2 reduction proceeds via RHP. A consequence of this assumption is construction of the *R. rubrum* pyridine nucleotide oxidase system as comprising an RHP-reductase with the rapidly auto-oxidizable RHP acting as terminal oxidase when air is present. Thus, the same scheme formulated previously from studies of crude sonicates[3] appears to hold for the partially purified soluble electron transfer system described herein.

The fact that cytochrome c_2 is very sluggishly oxidized but very rapidly reduced even under aerobic conditions confirms the observations of SMITH on crude cell-free extracts of *R. rubrum* in which it was observed that succinate oxidation proceeded much more rapidly than oxidation of cytochrome c_2 (see ref. 5). As a working hypothesis, the role of cytochrome c_2 can be taken to be that of mediating oxidation of the electron transport chain by the photo-oxidant produced during photosynthesis.

The reaction scheme which is essentially a restatement of the one discussed by KAMEN AND VERNON[3] can be presented diagrammatically as follows:



In the scheme, reaction 1 is catalyzed by reduced coenzyme–RHP reductase. Under aerobic conditions, the resulting RHP in its reduced form can be oxidized rapidly by oxygen (reaction 2). Under anaerobic conditions, the RHP reduced by the reduced coenzymes can reduce cytochrome c_2 much more rapidly than under aerobic conditions, probably non-enzymically, as in reaction 3. Cytochrome c_2 in its reduced form, if added in excess to this system, could partially reduce RHP which exists wholly in its oxidized form under aerobic conditions because of its great auto-oxizability (reverse reaction 3). The reductant and oxidant produced by the photochemical apparatus of light-grown cells of *R. rubrum* in the light can be assumed to reduce and oxidize RHP and cytochrome c_2 by reactions 4 and 5.

Thus, RHP is accorded a key position in electron transport shunting reduction

TABLE II

FURTHER AMMONIUM SULFATE FRACTIONATION OF COENZYME – 2,6-DCPI REDUCTASE

Fractions (% saturation with Ammonium sulfate)	Total extinction				Total enzyme activities	
	E^* (mμ)	$E^{ox.}_{640\ m\mu}$	$E^{red.}_{550\ m\mu}$ RHP + cytochrome c_2	$E^{red.**}_{550\ m\mu}$ Cytochrome c_2	DPNH 2-2,6-reductase activity (units)	DPNH oxidase activity ($-\Delta E^{DPNH}_{340\ m\mu}$/min)
Original enzyme solution	1,074$_{(256)}$	—	—	—	64.4	0.220
55–60	32$_{(270)}$	0.040	0.176	0.016	17.1***	(±)
60–78	208$_{(259)}$	0.752	2.500	0.134	2.24	(±)
78–100	154$_{(259)}$	1.554	5.952	0.306	0.68	(—)
Supernatant§	420$_{(256)}$	0.480	6.112	4.770	0.00	(—)

[*] These values show extinctions at u.v. wavelengths at which each fraction shows absorption maximum.

[**] These values were calculated from the following equation:

$$E^{red.}_{550\ m\mu\ (cytochrome\ c_2)} = E^{red.}_{550\ m\mu\ (RHP\ +\ cytochrome\ c_2)} - E^{ox.}_{640\ m\mu\ (RHP)} \times 3.63$$

[***] This fraction contained 4.6 mg of protein, specific enzyme activity being 3.72.

[§] The enzyme solution saturated with ammonium sulfate was centrifuged in the cold at 18,000 rev./min for 30 min with the use of a Servall automatic superspeed centrifuge. Resulting supernatant had turbidity.

equivalents (electrons, or H-atoms) either through cytochrome c_2 to the photo-oxidant, or directly to oxygen. In this way, an enzymic basis is provided for the remarkable competitive interaction between light and dark metabolism in facultative photoheterotrophes—a phenomenon noted long ago by NAKAMURA[23] and others[24]. Alternative explanations based on inactivation of light-sensitive components have been proposed, the most recent being given by VERNON[25] in connection with his observations on light suppression of DPNH-oxidase activity in *R. rubrum* chromatophores.

The present results suffer from the limitations imposed by the relatively weak activities obtained despite the mild character of the procedures employed. It will be necessary to continue efforts to obtain preparations of greater activity before resuming systematic studies to test further the hypothesis presented regarding the role of haem proteins in the metabolism of the photoheterotrophes. Such efforts will involve use of a variety of procedures based on more care in avoidance of extremes in pH and temperature, as well as more attention to removal of salts and traces of heavy metals.

ACKNOWLEDGEMENTS

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THE BINDING OF MAGNESIUM TO MICROSOMAL NUCLEOPROTEIN AND RIBONUCLEIC ACID

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SUMMARY

^{28}Mg was used as a tracer in studying the Mg binding by microsomal RNAP (ribonucleoprotein) and RNA isolated from rabbit reticulocytes. Methods for following the exchange of Mg by dialysis with ^{28}Mg, for precipitating RNA or RNAP with its complement of bound Mg, and for extracting the bound Mg are described in this report. The combination of these methods provides appropriate material for chemical and radioisotopic studies of the binding of Mg to polynucleotides.

Data has been presented to indicate that almost all, if not all, the Mg of RNAP is bound by the RNA moiety. Removal of the protein component from RNAP uncovers about 30 % more Mg binding sites.

Preparations of RNAP isolated from buffer of low K:Mg ratio contain mostly intact 80 S particles and have a Mg:P molar ratio of 0.3, similar to the ratio in intact pea microsomal particles. Dissociation of the 80 S particles occurs when the Mg:P molar ratio of RNAP is lowered to about 0.2 by isolating RNAP in buffer of high K:Mg ratio.

INTRODUCTION

A substantial binding affinity between divalent cations and RNA, DNA, RNAP, DNAP and synthetic polynucleotides has been demonstrated in this and other laboratories[1–4]. There is evidence which indicates that the structural integrity of microsomal nucleoprotein particles extracted from pea seedlings is critically dependent on the attachment of an optimal number of magnesium ions in the particles[1]. Similar results have been obtained in ultracentrifugal studies on RNAP isolated from rat liver[5].

In view of the fundamental role of divalent cations in nucleoprotein structure, and presumably in nucleoprotein function as well, we undertook (a) to study the rate of exchange of Mg bound to RNA and RNAP with Mg free in solution, (b) to determine

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Abbreviations: P, phosphorus; RNAP, ribonucleoprotein; DNAP, deoxyribonucleoprotein; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloracetic acid; EDTA, ethylenediamine tetraacetate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; Poly A, polyadenylic acid; Poly U, polyuridylic acid.

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** Contribution No. 2530.

the chemical Mg content of RNA and RNAP as a function of free Mg concentration, (c) to characterize the distribution of bound Mg in the microsomal particles, and (d) to extend previous observations on the dependence of microsomal particle size and homogeneity on bound Mg.

MATERIALS AND METHODS

Preparation of microsomal particles

Microsomal particles were isolated from rabbit reticulocytes by differential centrifugation as described previously[6,7] with minor modifications. The reticulocytes were harvested in quantity from rabbits made anemic by daily injections of phenylhydrazine[8]. After one week of injections the reticulocytes constitute 80–90 % of the erythrocyte population[6]. Earlier studies demonstrated that these cells synthesize hemoglobin actively and that the isolated microsomal particles take up radioactive amino acids rapidly [6,8–10]. The reticulocytes were washed three times with Na–K–Mg buffer, frozen and stored until used. Leukocytes were removed during the washing process. To prepare microsomal particles, the reticulocytes were thawed and suspended in 2 volumes of cold solution A' (0.14 *M* KCl, 0.0015 *M* $MgCl_2$ and 0.01 *M* Tris buffer, pH 7.2). Three cycles of differential centrifugation in solution A' were used to isolate the microsomal particles. The final dilution with solution A' was adjusted to give a RNAP concentration of 12–17 mg/ml.

RNA was prepared from microsomal RNAP by the method of Ts'o *et al.*[11]. The method consists of diluting the RNAP with solution A' to a concentration of 5 mg/ml and adding 0.10 g of Na dodecyl sulfate (recrystallized twice from Dupanol-C, Du Pont Co.) and 0.5 ml *n*-butanol to 10 ml of the diluted RNAP solution. The reaction mixture is incubated at room temperature for 15 min. Two volumes of chloroform–amyl alcohol (3:1 v/v) are added to 1 volume of the reaction mixture and homogenized to form a uniform suspension. The homogenate is incubated at 38° for 15 min and then is centrifuged at 10,000 rev./min for 15 min at room temperature in a Servall centrifuge. The upper aqueous layer is aspirated with a syringe and needle from the aggregated protein layer which separates the lower organic phase from the upper aqueous phase. The RNA in the aqueous solution is then precipitated with 2 volumes of ethanol at 0°. The final concentration of RNA in solution A' was adjusted to 4–7 mg/ml. These solutions were stored at — 60° until used.

Ultracentrifuge techniques

A Model L Spinco was used for preparative ultracentrifugation. A Model E instrument with schlieren or u.v. absorption optics was utilized for analytical ultracentrifugation, as described previously[1]. The films were traced by a Double-Beam Recording Microdensitometer, Joyce Lobel Co., Newcastle upon Tyne, England.

Chemical analyses

Assays for RNAP and RNA were performed by u.v. absorption spectra with a Beckman DU spectrophotometer or a DK-2 ratio recording spectrophotometer and by phosphorus analyses according to the method of ALLEN[12]. A factor of 11 was employed for the conversion of weight of P to the weight of RNA. Protein content was determined by the biuret method of CORNELL *et al.*[13], employing bovine serum albumin as the standard. Dry weight was determined by dehydration at 60–65° *in vacuo* to constant weight. A modification of the method of ROBINSON AND RATHBUN[14] was used to measure Mg content. All samples to be analyzed for Mg were adjusted to a pH of 8.5 with NH_4OH and calcium was removed with 4 % $NH_4C_2O_4$. The samples were mixed with micro-magnetic stirrers during titration with EDTA in the cuvettes. Data collected to validate the Mg method are presented under RESULTS.

Radioactive tracer methods

Magnesium exchange studies were carried out with ^{28}Mg* using dialysis chambers in one set of experiments and serial ethanol precipitation in a second set. Three-compartment Lucite chambers, with sheets of Visking sausage casing interposed between each compartment, were used for the dialysis studies. The capacity of the center compartment was 10 ml and of the two outer compartments 5 ml each. 10 ml of solution A′ labeled with ^{28}Mg was added to the center chamber and 5 ml of either RNAP or RNA suspended in cold solution A′ to each of the outer chambers. The solutions were mixed by means of separate micromagnets in each chamber. The entire assembly was placed on a magnetic stirrer in the cold room at 3°. Successive 0.1-ml samples were withdrawn at intervals of 15, 30 and 45 min and 1, 2, 3, 5 and 8 h after filling the chambers. The experimental samples and triplicate 0.1-ml aliquots of the standards were plated on aluminum planchets, dried under an infrared lamp and assayed for radioactivity in a gas flow counter, Model 181, with a D 47 Micromil window detector equipped with an automatic sample changer (Nuclear-Chicago Corporation, Chicago).

Calculations

The fractional exchange of bound Mg with radioactive Mg was obtained from the residual radioactivity in the center chamber and the known volumes and concentrations of RNAP or RNA. This system conformed to the kinetics of a two-compartment or two-phase system which may be represented by:

$$N^*_1 = N^*_0 \left(\frac{N_2}{N_1 + N_2}\right) e^{-\lambda t} + N^*_0 \left(\frac{N_1}{N_1 + N_2}\right) \tag{1}$$

$$\lambda = n_r \left(\frac{N_1 + N_2}{N_1 - N_2}\right) \tag{2}$$

where N^*_1 = amount of ^{28}Mg in the center compartment at any time t; N^*_0 = amount of ^{28}Mg introduced in the center compartment at time 0; N_1 = moles of Mg in the center compartment; N_2 = moles of Mg in the outer compartments; n_x = unidirectional flux of Mg in moles/min. The flux values were obtained from the slope of a plot of $Ln\ (N^*_1 — N^*_{1-8})$ *vs.* time, where N^*_{1-8} was the total ^{28}Mg content of the center compartment after 8 h of equilibration. The 8-h exchangeable Mg content of RNAP or RNA was obtained from:

$$Mg^*_e = \left[N_1 \left(\frac{N^*_0}{N^*_1} - 1\right) - N_s\right] \frac{1}{W} \tag{3}$$

---

* ^{28}Mg is a β, γ emitter with a 21.3 h half-life which decays to ^{28}Al; the latter is a strong β, γ emitter with a 2.3 min half-life. The isotope was supplied as $MgCl_2$:HCl solution by the Brookhaven National Laboratory.

where Mg^*_e = 8-h exchangeable Mg content in moles/mg dry wt. of particle; N_s = moles of free Mg in solution A′ in the outer compartments; W = dry wt. in mg of RNAP or RNA in the outer compartments.

RESULTS

Ultracentrifugal patterns of RNAP and RNA

The reticulocyte RNAP isolated in solution A (0.14 M KCl, 0.001 M $MgCl_2$, 0.001 M Tris, pH = 7.5) invariably contained 79 S particles, as well as a 58 S component which comprised about 30–40 % of the total nucleoprotein. Earlier studies[15] suggested that the 58 S component is one of the sub-units liberated from the primary 79 S particles because solution A is deficient in Mg. As shown in Fig. 1a, microsomal particles isolated in solution A′, which had a Mg content of 0.0015 M, also consisted of three components, 58 S, 77 S and aggregates. Reduction of the KCl concentration to 0.05 M (solution C, 0.05 M KCl, 0.0015 M $MgCl_2$, 0.001 M Tris, pH 7.5), however, markedly altered the pattern, as seen in Figs. 1c and d. The 58 S component was no longer present, and the preparation consisted solely of 79 S particles, its dimer (114 S) and other aggregates. Moreover, considerable evidence has been obtained to indicate that the 114 S component is formed from the 80 S species[15].

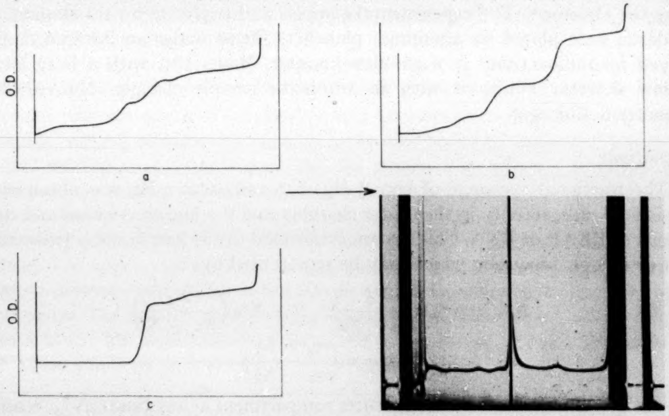

Fig. 1. Ultracentrifugal diagrams of reticulocyte microsomal particles. Sedimentation proceeds from right to left. 1a, b, and c are u.v. absorption diagrams; 1d is the schlieren diagram. a, particles prepared and centrifuged in 0.14 M KCl, 0.0015 M $MgCl_2$, and 0.001 M Tris (pH 7.5) (solution A) at 24.6°; b, microsomal RNA in 0.1 M Na-acetate, 0.001 M $MgCl_2$ (pH 5.5) at 9.2°. RNA was isolated from particles prepared in solution A; c, particles prepared and centrifuged in 0.05 M KCl, 0.0015 M $MgCl_2$, and 0.001 M Tris (pH 7.5) at 27.8°; d, same material as 1c at 25.5°.

The RNA pattern illustrated in Fig. 1b is similar to those described previously, except that the 13 S component is absent[11]. Most of the material sediments as 18 S and 30 S species, which appear to be reasonably homogeneous.

RNA: Protein ratio of the reticulocyte microsomal particles

The ratio of the weight of RNA to the weight of RNAP as estimated by determination of the P content and the dry weight of the microsomal particles was 0.495. The ratio of RNA : RNAP calculated from measurement of the P and protein contents was 0.481–0.510. In all subsequent calculations, therefore, we assumed a RNA : protein ratio of 1.00 as characteristic of reticulocyte ribosomes. This ratio agrees closely with the previously reported estimate[6].

Exchange of Mg bound to RNAP and RNA with free ^{28}Mg

The exchange of ^{28}Mg across the dialysis membrane in the three-chamber system at 3° averaged 0.12 μmole/min or $8 \cdot 10^{-3}$ μmole/min/μmole of Mg in the solution A′ in the outer chambers (Table I). The apparent flux of bound Mg from the RNAP particles with solution A′ Mg averaged 0.20 μmole/min or 0.65 μmole/min/μmole of RNAP Mg. The flux of RNA Mg was about 10% faster. The 8-h exchangeable Mg pool of RNA averaged about 2.5 times the RNAP pool size. Taken together, these data suggest that removal of the protein unblocks additional binding sites for Mg in the polynucleotide since the RNA : protein ratio is close to 1 : 1 in RNAP. The similarity in the flux rate constant of the dialysis membrane and of the particles suggested that the calculated particle fluxes reflect hindrance to the passage of Mg across the membrane. Consequently, we eliminated the membrane from the system and used a system of serial ethanol precipitation as an alternative method for estimation of RNAP and RNA Mg exchange rates.

TABLE I

EXCHANGE OF ^{28}Mg WITH ^{24}Mg OF RETICULOCYTE RNAP AND RNA AT 3°

Sample	*8-h exchangeable Mg* *μmole/mg*	*Flux of bound Mg with solution A′ Mg* *μmole/min*	*Fractional Mg exchange × 10^{-3}* *μmole/min/μmole*
RNAP	0.15	0.202	6.73
RNAP	0.15	0.197	6.31
RNA	0.36	0.235	6.87
RNA	0.40	0.210	6.60
Control solution A′	—	0.113	7.69
Control solution A′	—	0.137	9.07

Ethanol isolation of reticulocyte RNAP and RNA

The validity of ethanol precipitation in the recovery of RNAP and RNA with an unmodified Mg content was evaluated as follows: The measured ^{28}Mg content of the precipitates obtained by treatment of RNAP or RNA solution with absolute ethanol (1 volume to 3 volumes) at 3°, followed by three ethanol washes, was compared with the calculated ^{28}Mg content of these constituents after equilibration for 8 h in the dialysis chambers. As shown by the data listed in Table II, this procedure results in the recovery of particles substantially free of adsorbed Mg and no loss of intrinsic Mg content. This technique, therefore, makes it possible to study the flux of RNAP and RNA Mg, as well as the exchangeable Mg content, by direct incubation in solution A′ labeled with ^{28}Mg. The exchange of RNAP and RNA Mg was determined by dividing these solutions into seven 1-ml aliquots, adding ^{28}Mg at 3° and precipitating the polynucleotides with 3.0 ml of absolute ethanol serially at 30 min and 1,

TABLE II

^{28}Mg BOUND TO RNA OR RNAP AS MEASURED BY THE ETHANOL PRECIPITATION METHOD AND THE DIALYSIS EXCHANGE METHOD

Sample	*Specific activity*		*Ratio of S.A. of precipitate to S.A. calculated by exchange*
	By ETOH precipitation Counts/min/mg	*Calculated by exchange Counts/min/mg*	
Reticulocyte RNAP	544	502	1.08
Reticulocyte RNA	821	792	1.04
Reticulocyte RNA	1324	1254	1.05

TABLE III

^{28}Mg CONTENT OF RNAP AND RNA BY SERIAL ETHYL ALCOHOL PRECIPITATION

Sample	*Equilibration time (h)*	*^{28}Mg content*	
		Mean	*Range*
		μmole/mg	
RNAP	0.5	0.16	
RNAP	1–10	0.23	0.22–0.25
RNA	0.5	0.76	
RNA	1–10	0.80	0.75–0.90

2, 3, 6 and 10 h. The precipitates were packed by centrifugation and washed 3 times with absolute ethanol. The ethanol was decanted and the last traces were evaporated in a gentle air stream. The precipitates were redissolved in 1.0 ml of distilled water; 0.4-ml aliquots were then plated, dried under an infrared lamp and assayed for radioactivity as detailed above. The results in Table III show that there is almost 100 % exchange after incubation for 30 min. The mean exchangeable Mg content was 0.23 and 0.80 μmole/mg for RNAP and RNA, respectively, or 1.5 to 2.0 times greater than the Mg^*_e values obtained with the chamber dialysis technique. To determine whether hindrance to Mg transport by the dialysis membrane results in incomplete equilibration, we then measured the bound Mg content of RNAP and RNA by a direct chemical method. The cold ethanol precipitation method offers a technique for isolating these substances from the buffer Mg without artifacts. The next step, however, required release of 100 % of the Mg from the bound state and away from interfering phosphate ions. Earlier studies suggested that this might be achieved with either 8 % TCA or 0.05 *M* Na EDTA[1].

Recovery of ^{28}Mg in TCA or EDTA supernatants

Either RNAP or RNA was equilibrated with ^{28}Mg-labeled solution A′ in the dialysis chamber for 10 h. The labeled RNAP or RNA was recovered by ethanol precipitation as outlined previously and resuspended in distilled water; aliquots were then taken for radioassay or treatment with TCA or EDTA. The TCA method consisted of adding 1.0 ml of 16 % TCA to 1.0 ml of RNAP or RNA solution, incubating at 3° for 30 min, centrifuging to remove the precipitate and plating 0.5-ml aliquots of the supernatants for radioassay. The EDTA method consisted of adding 4 ml of

0.05 M EDTA (pH 7.65) to 1.0 ml of RNAP or RNA solution, incubating at 3° for 10 h, centrifuging at 100,000 × g for 8 h and plating 1.0 ml aliquots of the supernatants for radioassay. As shown in Tables IV and V, approx. 100% of the RNAP or RNA ^{28}Mg was recovered in the supernatants. The TCA precipitates and EDTA-treated pellets had negligible radioactivity when assayed directly. Because of its convenience we used the TCA method for subsequent chemical measurements of Mg content.

TABLE IV

RECOVERY OF ^{28}Mg IN TRICHLORACETIC ACID SUPERNATANTS

Sample	^{28}Mg activity in RNAP or RNA	^{28}Mg activity in TCA supernatant	Fractional recovery
	Counts/min		
Reticulocyte RNA	3951	3970	1.00
Reticulocyte RNA	6705	7350	1.09
Reticulocyte RNAP	4014	4108	1.02

TABLE V

RECOVERY OF ^{28}Mg IN ETHYLENEDIAMINE TETRAACETATE SUPERNATANTS

Sample	^{28}Mg activity	^{28}Mg activity in EDTA supernatants	Fractional recovery
	Counts/min		
Reticulocyte RNA	2743	2656	0.97
Reticulocyte RNA	7766	7286	0.94

Mg content of reticulocyte RNAP and RNA

To validate the ROBINSON AND RATHBUN[10] method for Mg analysis, we established that (a) the relation between the EDTA titration volume and the Mg concentration of standard solutions is linear and goes through the origin, (b) the titration volume is independent of the anion, yielding the same values for $MgSO_4$ and $MgCl_2$ at equal molarities, (c) addition of up to 25% Ca or Ba or Sr to the Mg solutions has no detectable effect on the final titration volume, (d) addition of TCA to a final concentration of 8% and neutralizing with NH_4OH has no effect on the titration volume, and (e) the reproducibility of measurements of both standards and unknown samples of biological material (human plasma) is ± 2.8% (standard deviation expressed as % of the mean). Aliquots of RNAP or RNA in solution A′ and RNAP in solution C (0.05 M KCl, 0.0015 M $MgCl_2$, 0.001 M Tris, pH 7.5) were subjected to ultracentrifugal analysis as described above. RNAP or RNA was isolated from these solutions by cold ethanol precipitation and the washed precipitates were resuspended in distilled water. The nucleotide content of the samples was estimated from measurements of u.v. absorbancy and P analysis. Additional aliquots were treated with TCA and the supernatants were analyzed for Mg content as described above. The results of chemical analysis of distilled water solutions of reticulocyte RNA and RNAP are summarized in Table VI. RNA bound from 2.8–3.4 times as much Mg as RNAP per unit weight of particle, and about 30 % more than the RNA in ribosomes. These data agree closely with values obtained in the experiments on ^{28}Mg exchange (see Table III). The fact that similar results were obtained in the dialysis experiments (Table II) supports the view that removal of the protein uncovers additional binding sites in the polynucleotide and justifies the supposition that at physiologic Mg concentrations, all or nearly all of the Mg is bound to the RNA moiety. To evaluate the latter possibility we measured the recovery of ^{28}Mg bound to RNAP in RNA isolated on fractionation of the RNAP and also compared the dependence of RNAP and of RNA bound Mg on free Mg concentration.

TABLE VI

CHEMICAL ANALYSIS OF RETICULOCYTE RNA AND RNAP

Sample	*Concentration* mg/ml*	*Phosphorus µmole/mg*	*Magnesium µmole/mg*	*Mg/P Mole ratio*	
					mean
RNA (solution A)	2.5	3.00	0.72	0.24	0.23
RNA (solution A)	3.3	3.00	0.67	0.22	
RNAP (solution A)	12.4	1.40	0.23	0.17	0.18
RNAP (solution A)	6.0	1.40	0.27	0.19	
RNAP (solution C)	5.7	1.38	0.43	0.31	

* Determined by u.v. absorption spectra.

Reticulocyte microsomal particles suspended in solution A′ were equilibrated with ^{28}Mg-labeled solution A′ in the dialysis chamber for 8 h as described above. The ^{28}Mg content of the RNAP was calculated from the residual activity of the solution A′ in the center chamber, and the RNAP solution was recovered from the outer chambers. The nucleoproteins were split into an RNA and a protein fraction by the method of Ts'o *et al.*[11] as detailed above and the isolated RNA was counted. All of the RNAP-bound ^{28}Mg was recovered in the RNA fraction within the limits of the error of the methods (Table VII).

To test whether RNA could account for all the Mg bound to RNAP over a wide concentration range, the following reaction mixtures were prepared:

ml of 1.5 M $MgCl_2$	*ml of solution A′ ^{28}Mg*	*ml of RNAP or RNA*	*Total volume in ml*	*Mg concentration in µmole/ml*
0	0.5	0.5	1.00	1.5
0.005	0.5	0.5	1.005	9.0
0.010	0.5	0.5	1.010	16.4
0.025	0.5	0.5	1.025	38.8
0.050	0.5	0.5	1.050	76.0

At Mg concentrations greater than 25 µmoles/ml the solutions invariably were cloudy.

The reaction mixtures were incubated at 3° for 11 h, at which time 3 ml of cold absolute ethanol was added to each tube; RNAP and RNA were then recovered in distilled water as described above. The radioactivity of each sample was assayed; the phosphorus content and u.v. absorption were measured; the results are plotted

TABLE VII

ASSOCIATION OF ^{28}Mg WITH RNA OF RETICULOCYTE RIBOSOMES

8-h exchangeable Mg of RNAP	*Specific activity of RNAP*	*Measured specific activity of RNA*	*Calculated specific activity of RNA*	*Ratio of measured to calculated specific activity*
μmole/mg	*Counts/min/mg*			
0.12	185	390	370	1.05
0.15	502	903	1004	0.90

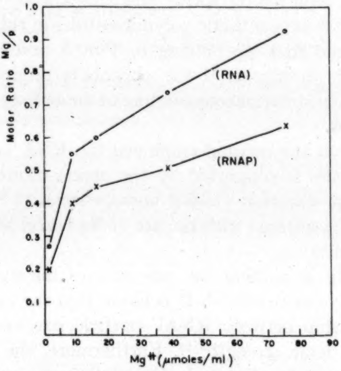

Fig. 2. The molar ratio of Mg:P bound to RNA (○—○) or to RNAP (×—×) as a function of free Mg concentration. For conditions of incubation with ^{28}Mg and details of separation of labeled particles from the incubating medium by ethanol precipitation, see text.

in Fig. 2. The similarity in the shapes of the curves supports the idea that RNA provides all the binding sites for Mg in the nucleoprotein. These data also confirm the greater binding capacity of RNA when freed of protein; at a Mg ion concentration of 0.076 *M*, RNA bound 0.93 moles of Mg/mole of P, whereas RNAP bound 0.64 mole/mole.

DISCUSSION

The suggestion in a previous report[1] that virtually all the Mg in RNAP is bound by RNA with little or no binding by protein is now supported by four lines of evidence: (a) the binding capacity of free microsomal RNA for Mg can account for all the binding capacity of RNAP, (b) the kinetics of the exchange of Mg in both RNA and RNAP is very similar, (c) the ^{28}Mg bound by RNAP can be quantitatively recovered in the RNA moiety, and (d) that Mg:P molar ratios of RNA and RNAP show a parallel dependence on the Mg concentration of the buffer. Moreover, the data (Tables II, III, VI and Fig. 2) indicate that free RNA binds about 30 % more Mg than the RNA in RNAP. Thus, the protein in RNAP appears to block about 30 % of the sites which are available for Mg binding in RNA. Although the binding sites for Mg in RNA have not been definitely identified, previous investigators have inferred[2,3] that

Mg is bound to the negatively charged phosphate groups. There are indications, however, that in other nucleotides, namely DNA, ATP and ADP, the nitrogen bases may participate in Mg binding by a chelation mechanism[16,17]. If the phosphate group in RNA is the only major binding site for Mg, it may be inferred that the basic groups of the protein in RNAP combine with the phosphate groups of RNA to form the nucleoprotein complex of the intact microsomal particle[18].

The Mg:P molar ratio of RNA and RNAP exceeds 0.5 when the Mg concentration of the medium is greater than 0.01 M (see Fig. 2). WIBERG AND NEUMAN[3] assumed that the maximum Mg:P molar ratio would be 0.5, based on the data on divalent cation binding to DNA reported by KATZ[19] and SHACK *et al.*[20]. Our data also indicate that native RNA differs from synthetic polynucleotides in this respect, since FELSENFELD AND HUANG[2] found that Mg binding to Poly A and Poly U is limited to 0.5 moles/mole of polymer phosphate. If the phosphate groups constitute the major binding sites for Mg, then simultaneous binding of anion may also occur in ribosomal particles or native RNA.

The assumption that the method employed for RNA separation yields reproducible and intact species is supported by the observations of HALL AND DOTY[21]. Our method of RNA preparation yielded homogeneous 18 S and 30 S components (Fig. 1b), while the latter authors with the use of Na lauryl SO_4 and phenol obtained 18 S and 28 S components.

The key role of Mg in holding the sub-units of the native ribosomal particle together is confirmed by our results[1,5]. It is likely that the binding of Mg to RNAP, as well as the relationship between RNAP particle size and Mg concentration, is dependent on pH and ionic strength[5,22]. Furthermore, the K:Mg ratio is critical in determining the magnitude of particle dissociation at neutral pH. In solution A' (K:Mg molar ratio of 95), RNAP has a Mg:P ratio of 0.18, and a substantial amount of the 60 S sub-unit is present in the solution (Fig. 1a). Our unpublished data show a dissociation of 80 S particles in solution A' and preferential disintegration of the smaller sub-units (30-40 S). In solution C (K:Mg molar ratio of 33), there is no dissociation of 80 S particles into sub-units, and the Mg:P ratio of RNAP is 0.3, a value similar to that reported for intact pea ribosomal particles[1]. Apparently both reticulocyte and pea ribosomes require a Mg:P ratio of 0.3 to stabilize the 80 S particles.

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STUDIES ON THE ENZYMIC DECOMPOSITION OF UROCANIC ACID

II. PROPERTIES OF PRODUCTS OF UROCANASE REACTION

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SUMMARY

1. Urocanase has been purified about 150 fold from beef liver by classical enzyme fractionation procedures. The purified enzyme is free of imidazolone propionic acid oxidase and hydrolase activities.

2. Evidence indicating that the product of urocanase reaction is the suspected 4(5)-imidazolone-5(4) propionic acid has been obtained by comparison of its spectral and chemical properties to other known imidazolones.

3. Imidazolonepropionic acid is labile to oxygen but no hydantoin-5-propionic acid is formed by this nonenzymic oxidative reaction. It is also nonenzymically converted to formylisoglutamine, which was isolated and characterized by its physical and chemical properties.

4. The enzymic formation of formiminoglutamic acid from imidazolonepropionic acid was demonstrated by a coupled assay procedure in crude extracts of liver of various animal species and also in bacteria.

INTRODUCTION

Previous studies[1-6] have established the probable sequence of reactions for the enzymic conversion of urocanic acid to formiminoglutamic acid and its nonenzymic conversion to formylisoglutamine as shown below:

$$\text{Urocanic acid} + H_2O \xrightarrow{\text{Urocanase}} \text{Imidazolonepropionic acid} \qquad (1)$$

$$\begin{tikzcd} \text{Imidazolonepropionic acid} + H_2O \arrow[r, "\text{Imidazolonepropionic acid hydrolase}"] \arrow[dr, "\text{Nonenzymic}"'] & \text{Formimino-L-glutamic acid} \qquad (2) \\ & \text{Formylisoglutamine} \qquad (3) \end{tikzcd}$$

The present report deals with the purification and separation of urocanase from imidazolonepropionic acid hydrolase, the properties of the product of reaction (1) (imidazolonepropionic acid), the unequivocal identification of formylisoglutamine and the further study of enzyme reaction (2).

EXPERIMENTAL

Materials

Urocanic acid was purchased from the California Corporation for Biochemical Research. Formiminoglutamic acid was prepared by the method of RABINOWITZ AND TABOR[7]. Formyl-L-glutamic acid and formyl-L-glutamine were prepared by the method of BOREK AND WAELSCH[2]. Formyl-L-isoglutamine was kindly furnished by REVEL AND MAGASANIK. Isoglutamine was made from carbobenzoxy-L-glutamic acid by the method of BERGMANN AND ZERVAS[8], and carbobenzoxy-L-glutamic anhydride by the method of LEQUESENE AND YOUNG[9]. A sample of isoglutamine was also kindly furnished by Dr. G. WOLF. Tetrahydrofolic acid was prepared by the method of RABINOWITZ AND PRICER[10]. L-Hydantoin-5-propionic acid was prepared by the method of DAKIN[11].

Chromatographic methods

For the paper chromatographic comparison and identification of the various metabolites of urocanic acid, the solvent systems described by REVEL AND MAGASANIK[5] were adopted. Radioactivity on chromatograms was detected with a recording, thin end window Geiger-Müller Counter for scanning paper strips.

Glutamic acid, isoglutamine, and formiminoglutamic acid were detected on paper chromatograms by dipping the paper in a 0.2 % solution of ninhydrin in acetone and heating. Formiminoglutamic acid was also detected with a ferricyanide–nitroprusside reagent[12] and by the enzymic method of TABOR AND WYNGARDEN[13]. Imidazole compounds were located with the diazotized sulfanilic acid reagent of AMES AND MITCHELL[14]. N-substituted amino acids (*i.e.*, formylisoglutamine, formylglutamine and formylglutamic acid) were detected by the modified starch-iodide procedure of PAN AND DUTCHER[15].

Urocanic acid was separated from histidine by ion exchange chromatography with Amberlite IRC-50 (XE-64) resin, which was processed as described by HIRS[16]. An 18 × 2.5 cm column was packed under gravity with the Na^+ form of resin and buffered at pH 4.8 by passing through 250 ml of 1 M sodium acetate buffer, pH 4.8, then washing with water until the washings were neutral. A mixture of D-histidine and urocanic acid in 30 ml of solution was gradually applied to the column over a period of one hour, followed by 25 ml of water. A gradient elution device containing 0.1 N acetic acid in the reservoir and 250 ml of water in the mixing flask was attached to the column and 3-ml fractions at the rate of 0.6 ml/min were collected. Approximately 90 % of the urocanic acid was recovered as a single peak in fractions 40 to 50. Most of the D-histidine was later recovered by passing 1 N acetic acid through the column. The urocanic acid was chromatographically homogenous in several solvent systems[4, 5] and showed only one radioactive spot.

For the separation of the enzymic and nonenzymic degradation products of the urocanase reaction, ion exchange chromatography on a Dowex 1-acetate column (200–400 mesh, X-8) was used. The gradient elution device described above, but containing 0.3 N acetic acid in the reservoir, was employed. Fractions from ion exchange chromatography and other reaction mixtures were routinely subjected to high voltage paper electrophoresis at pH 3.5 and pH 6.4 at 2 kV and 40–50 mA in an apparatus described by MICHL[17]*. Formylglutamic acid, formylisoglutamine, glutamic acid and isoglutamine were separable at pH 6.4. Isoglutamine could be distinguished from glutamine at pH 3.5 but not at pH 6.4.

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* We wish to thank Dr. D. GROSS of this department for various suggestions in the use of the electrophoresis apparatus.

Enzyme fractions used in the present study

Histidase was prepared from extracts of *Pseudomonas fluorescens* cells by the method of TABOR AND MEHLER[18], except that the heat denaturation of inactive protein was carried out at 70–72° for 5 min instead of 78–83° for 15 min. Formiminoglutamic transferase was prepared by the method of TABOR AND WYNGARDEN[13] with the slight modification (for greater reproducibility) that the first ammonium sulfate precipitate "step B" was dissolved in 0.05 *M* potassium phosphate buffer, pH 7.2, dialyzed against 10 volumes of the same buffer for 16 h and further fractionated with solid ammonium sulfate. Fractions containing most of the formiminoglutamic transferase activity were pooled, dissolved in 0.05 *M* potassium phosphate buffer, pH 7.2, frozen and kept at —20° until needed for further use. In some preparations these fractions contained substantial quantities of imidazolonepropionic acid hydrolase, as determined by a coupled assay procedure. The formiminoglutamic transferase activity of these fractions could be demonstrated even after three months of storage at —20°.

In the initial stages of enzyme purification protein was determined by the biuret method of ROBISON AND HOGDEN[19] and in the later stages by the u.v. absorption method of WARBURG AND CHRISTIAN[20]. Units of activity and specific activity of histidase and urocanase preparations are those of TABOR AND MEHLER[18].

All optical measurements were made in a Cary Model 14 recording spectrophotometer.

RESULTS

Purification of urocanase

The method of purification of this enzyme reported by FEINBERG AND GREENBERG[6] has been considerably modified as described below. All operations were carried out at 0–5°.

Two portions of 500 g each of fresh beef liver were homogenized with 1500 ml of 0.25 *M* sucrose containing 0.005 *M* EDTA (ethylendiaminetetraacetate) at pH 7.2 for 15 sec at medium speed and 45 sec at full speed in a large Waring blendor of 1 gallon capacity. The pooled homogenate was centrifuged at 2700 × *g* for 30 min in a Servall refrigerated centrifuge. The supernatant fluid was filtered through one fold of cheese cloth into a 4-l Erlenmeyer flask, yielding a turbid filtrate with a volume of 3500 ml. A small measured volume of this fluid was centrifuged at 25,000 × *g* for 30 min in a Spinco preparative ultracentrifuge and urocanase activity determined on the clear supernatant fluid. The total activity in the original homogenate was computed from this determination. In many instances the urocanase activity of various beef liver homogenates was extremely low but the activity increased and could be detected as purification progressed.

To each liter of the turbid filtrate 100 g of a 1:1 mixture of Bentonite (the brand appears immaterial) and Hyflo-Supercell (Johns Mansville Corporation) were added and thoroughly mixed at medium speed for 1 to 2 min in a large Waring blendor. The thick suspension was left to stand for 1 h at approx. 0° in an ice bath, then centrifuged at 10,000 × *g* for 1 h and the opalescent supernatant fluids (volume 1800 ml) were pooled. 564 g of ammonium sulfate were added gradually over a period of 1 h or more. The pH was maintained at 6.8–7.2 by addition of 3 *N* NH_4OH. (pH measurements were made on a 1:10 sample dilution). The solution was left to stand for at least 12 h in an ice bath and later centrifuged at 10,000 × *g* for 1 h. The precipitate was dissolved in 0.1 *M* potassium phosphate buffer, pH 7.2, and dialyzed against 2 l of the same buffer containing 0.001 *M* EDTA. The buffer was changed after 8 to 10 h. After 16–20 h of dialysis, the protein solution was centrifuged at 15,000 × *g* to remove insoluble material and the supernatant fluid diluted with 0.1 *M* potassium phosphate buffer, pH 7.2, to give a protein concentration of 20 mg/ml. This solution was further fractionated with solid ammonium sulfate starting from 0.2 saturation and raising the concentration by intervals of 0.05 saturation. Most of the activity was found between 0.2 and 0.5 saturation of ammonium sulfate. Fractions having a specific activity of over 60 were pooled, dissolved in the same buffer and dialyzed as described above for 12 h.

The dialyzed protein was diluted approximately to a protein concentration of 10 mg/ml and subjected to fractional negative adsorption on calcium phosphate gel as described below.

An enzyme preparation in 90 ml containing 928 mg of protein was stirred with 930 mg of packed calcium phosphate gel[21] for 45 min in an ice bath and then centrifuged at 10,000 × *g* for 15 min. The supernatant fluid was assayed for activity and again stirred with an amount of gel equivalent to the total protein for 30 min and centrifuged. The process was repeated several times and the final supernatant fluid from the gel was fractionated with ammonium sulfate. A considerable quantity of inactive protein is retained on the packed gel. On elution with 0.5 *M* potassium phosphate buffer, pH 7.2, enzyme of low specific activity could be recovered.

Results of a typical fractionation procedure are shown in Table I.

TABLE I

PURIFICATION OF UROCANASE

Fraction	*Total protein mg**	*Specific activity*	*Total activity*	*Yield*
Homogenate	212,500	3.2	680,000	100
Bentonite supernatant fluid	15,400	32.5	500,500	73.6
1st ammonium sulfate precipitate	5,000	76.8	384,000	56.5
Pooled ammonium** sulfate fractions	3,100	92.0	285,200	42.0
Pooled ammonium*** sulfate fractions	930	70.0	65,100	100
1st gel supernatant	492	105.5	51,810	79.5
2nd gel supernatant	192	216.0	41,500	63.7
3rd gel supernatant§	82.5	273.0	22,530	34.6
0.3–0.4 saturation ammonium sulfate precipitate	32.0	440.0	14,080	21.6

* Protein of upper 4 purification steps was determined by biuret method and of lower 5 purification steps by u.v. absorption.

** Approximately 25 to 45% of the original activity is recovered at this stage, depending on the initial activity of the homogenate.

*** Pooled fractions of a different enzyme preparation from above, yield set at 100 for comparison.

§ Gel fractionation invariably gave 2 to 4 fold purification. The specific activity listed was obtained only once.

Highly purified enzyme fractions (about 150 fold enrichment, specific activity of 400 or more) show in addition to the usual absorption maximum at 280 mμ an inflexion at 290 mμ and end absorption in the spectral range of 350–450 mμ. These fractions are free of imidazolonepropionic acid hydrolase and imidazolonepropionic acid oxidase activities. Other properties of the enzyme are similar to those described earlier by FEINBERG AND GREENBERG[6]. The activity of the enzyme was unchanged after treatment with Norite or Dowex 1-acetate or EDTA. Considerable loss of activity occurred on treatment with protamine sulfate.

Preparation of [2-^{14}C]urocanic acid

[2-^{14}C]urocanic acid was prepared from DL-[2-^{14}C]histidine by a modification of the large scale preparative procedure of MEHLER, TABOR AND HAYAISHI[22].

Approximately 20,000 units of histidase in 3 ml of water was added to a mixture of 200 μmoles of DL-[2-^{14}C]histidine (dissolved in 10 ml of water and adjusted to pH 9.2), 140 μmoles of sodium thioglycolate and 400 μmoles of sodium pyrophosphate buffer, pH 9.2, in a total volume of 25 ml and left at room temperature for 3 h, with occasional stirring. The reaction mixture then contained 98 μmoles of urocanic acid, as determined spectrophotometrically. The reaction was terminated by adding 0.3 ml of glacial acetic acid and heating in a boiling water bath for 10 min. The denatured protein was removed by centrifugation and the supernatant fluid adjusted to pH 4.8–5.0. The urocanic acid was isolated from the solution by ion exchange chromatography on Amberlite IRC-50 (XE-64) as described previously.

Urocanic acid + H_2O → imidazolonepropionic acid

In order to determine whether or not this reaction proceeds to completion, radioactive urocanic acid was incubated with urocanase and reisolated at the end of the reaction by addition of carrier urocanic acid.

The reaction mixture contained 200 μmoles of potassium phosphate buffer, pH 7.2, 0.294 μmoles of urocanic acid (68,600 counts/min) and 260 units of urocanase of specific activity 400, in a total volume of 3 ml, in a spectrophotometric cuvette. The blank contained all components except urocanic acid. The O.D. at 277 mμ dropped to a constant value in 10 min. The contents of the cuvette were then quantitatively transferred to a chilled graduated centrifuge tube and rinsed with 3 ml of water. The reaction was terminated by addition of 0.4 ml of 10% perchloric acid, the denatured protein removed by centrifugation, the pH adjusted to 7 with 1 *N* KOH, and the volume made up to 8 ml. Aliquots were taken for determination of radioactivity and 200 mg of urocanic acid·2 H_2O were added as carrier and dissolved by heating in a boiling water bath. The solution was left overnight in the refrigerator. The crystals were separated from the supernatant fluid and recrystallized several times from hot water. After two recrystallizations the specific activity remained constant. The results of several experiments showed that 98–99% of the urocanate was utilized in the enzyme reaction.

Properties of imidazolonepropionic acid

Stability and spectral characteristics: In the beginning of these studies the only known characteristic of the product of the urocanase reaction was its absorption maximum of 264 mμ at pH 7.2 to 7.6. In order to isolate the "264 mμ product" it was necessary to deproteinize the reaction mixture, as traces of protein seriously interfered with spectrophotometric measurements. Only deproteinization with perchloric acid was found suitable for the desired purpose and to give reproducible results. Even when purified enzyme preparations of urocanase were used, considerable interference in the u.v. region by acid-soluble nucleotides was noticed. Consequently suitable blanks were run in all experiments. A typical experiment is described below.

The reaction mixture in matched Quartz spectrophotometric cuvettes contained 100 μmoles of sodium phosphate buffer, pH 7.6, and 350 units of urocanase of specific activity 70 in a total volume of 2.9 ml. 0.4 μmole of urocanic acid in 0.2 ml of water was added to one of the cuvettes, while 0.2 ml of water was added to the blank cuvette. The O.D. at 277 mμ dropped to a constant value in 8 min. The spectrum of the product was obtained by differential u.v. spectrophotometry and the contents of each cuvette was quantitatively transferred to a chilled glass centrifuge tube containing 0.1 ml of 30 % perchloric acid. After centrifugation at 3° to remove denatured protein, 2.5 ml of the clear supernatant fluid was pipetted from each tube into separate cuvettes and diluted to 3 ml with water, or 1 ml of undiluted aliquots were directly used in 1.5 ml cuvettes. Approximately 15 min were required to reach this stage after termination of the reaction. Various additions were made to each cuvette and the difference spectrum recorded at short time intervals. The difference spectral changes observed in such an experiment are shown in Fig. 1.

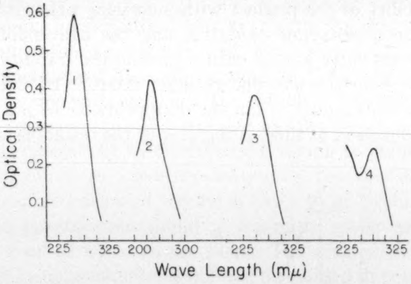

Fig. 1. Effect of acid and alkali treatment on product of urocanase reaction. Curve 1, spectrum of product of urocanase reaction, λ_{max}, 264 mμ; Curve 2, product after treatment with perchloric acid and dilution (pH 1.3), λ_{max}, 234 mμ; Curve 3, 30 sec after changing the pH to 9 with NaOH, λ_{max}, 250 mμ; Curve 4, 15 min after changing the pH to 9, λ_{max}, 275 mμ.

These experiments revealed that the absorption maximum of the "264 mμ product" changed to 234 mμ after perchloric acid treatment. In another experiment the perchloric acid extract was left under aerobic and anaerobic conditions for various periods at —20° and the absorption spectrum determined at different time intervals. Under aerobic conditions the product showed a 5 % decomposition in 26 h at —20°, whereas under anaerobic conditions there was no decomposition. After 76 h about 5 % decomposition was noticeable in the product preserved anaerobically. Attempts were made to recover the "264 mμ product" by increasing the pH to the original value of 7.2 to 7.6. For this purpose the perchloric acid supernatant fluid containing the product was adjusted to known pH values with measured volumes of 2 *M* $(NH_4)_2HPO_4$ and the spectrum scanned immediately. The results are shown in Fig. 2.

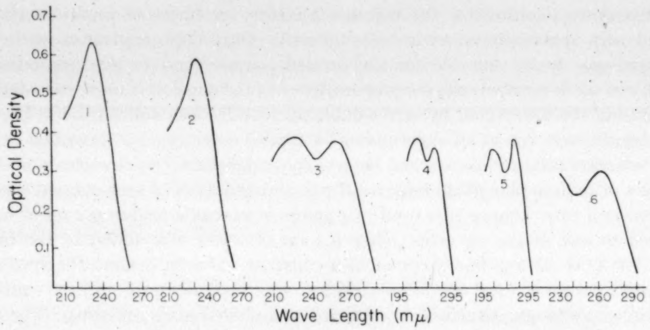

Fig. 2. Spectrum of product of urocanase reaction at various pH values. Curve 1, undiluted perchloric acid extract (pH 1.3), λ_{max}, 234 mμ; Curve 2, 30 sec after adjusting the pH to 2.9, λ_{max}, 234 mμ; Curve 3, 30 sec after adjusting the pH to 5.7, λ_{max}, 228 mμ, 260 mμ; Curve 4, after 5 min at pH 5.7, λ_{max}, 232 mμ, 265 mμ; Curve 5, 30 sec after adjusting the pH to 6.2, λ_{max}, 265 mμ; Curve 6, after 15 min at pH 6.2, λ_{max}, 260 mμ.

These experiments indicated that profound changes in the spectra took place, presumably because of the tautomerization of the imidazolone ring by acid-alkali treatment. The instability of the product with increasing pH values is possibly due to its susceptibility to nonenzymic oxidation and the irreversible opening of the imidazolone ring. Consequently kinetic data regarding the stability of the substance was obtained directly from the enzymic reaction mixture by determining the degradation of the "264 mμ product" from the disappearance of the absorption maximum at 264 mμ as a function of time. A log plot of the results is shown in Fig. 3.

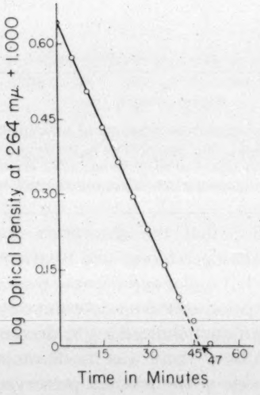

Fig. 3. Kinetics of nonenzymic degradation of "264 mμ product". The reaction mixture contained 200 μmoles of potassium phosphate buffer, pH 7.2, 400 units of urocanase of specific activity 123 and 0.4 μmoles of urocanic acid in a total volume of 3 ml. The blank contained all components except urocanic acid. The rate of nonenzymic degradation of "264 mμ product" was followed after the completion of urocanase reaction (*i.e.*, 12 min) for a further period of 1 h.

The nonenzymic degradation of the "264 mμ product" clearly follows first order kinetics with a velocity constant of 0.0309 min^{-1} and half life of 22 min at 27° at pH 7.2. The rate of nonenzymic degradation of the "264 mμ product" was independent of enzyme concentration, since addition of more enzyme did not cause a significant change in the rate.

Molar extinction coefficient: Isotope dilution experiments have indicated that virtually all the urocanate is utilized in the enzymic reaction and in previous work[6] it was shown that "264 mμ product" appears without time lag. On this basis a value for $E_{\max}$ of 3980 ± 300 was estimated from data of a number of experiments at pH 7.2. The $E_{\max}$ at 264 mμ did not vary significantly in the pH range of 6.3–8.6. In these experiments a large excess of enzyme was used, since the pH optima of the enzyme is between 6.8 to 7.6. No corrections have been made in the $E_{\max}$ value, for the nonenzymic degradation of the "264 mμ product" during its formation from urocanate. REVEL AND MAGASANIK[5] have also reported an approximate $E_{\max}$ value of 4000 for the product of the urocanase reaction.

Susceptibility of "264 mμ product" to oxidizing agents: The "264 mμ product" is extremely responsive to the action of oxidizing agents such as H_2O_2 or potassium persulfate. When small amounts of H_2O_2 or persulfate were added to the spectrophotometric cuvettes containing the product, the absorption maximum at 264 mμ disappeared instantaneously. No well defined products could be isolated from the oxidation reaction.

While the above investigations were in progress BROWN AND KIES[23] and BROWN[24] reported that hydantoin-5-propionic acid was a metabolite of urocanic acid in the rat. Previous experiments[6] have shown that the "264 mμ product" participates in a non enzymic reaction with oxygen in the presence of ferricyanide or dichlorophenol indophenol. An experiment to test whether hydantoin-5-propionic acid is formed in this reaction was carried out by the isotope trapping technique.

To spectrophotometric cuvettes containing 100 μmoles of sodium phosphate buffer, 400 units of urocanase of specific activity 50 in a volume of 2.8 ml, 0.3 μmole of urocanic acid (52,500 counts/min) in 0.2 ml of water was added to one of the cuvettes and 0.2 ml of water to the blank. The reaction was complete in 10 min. 0.9 μmole of dichlorophenol indophenol dye was added in portions of 0.3 μmole each. The color of the dye was bleached within 2 to 4 min during the first two additions of the dye. The mixture was then transferred to a centrifuge tube, deproteinized with an equal volume of acetone and centrifuged. The residue was extracted once with 3 ml of acetone containing 0.2 ml of 2 *N* HCl. The combined supernatant fluids were kept in an ice bath. Four experiments were performed similarly. The final supernatant fluids were pooled together and concentrated to near dryness at 35–40°. 200 mg of L-hydantoin-5-propionic acid in 5 ml of water were added, warmed to 50° in a water bath, the solution cooled to room temperature and centrifuged. Aliquots were taken for determination of radioactivity and the bulk of the solution left overnight in a refrigerator. The crystalline hydantoin-5-propionic acid was freed of the mother liquors and recrystallized several times from ice cold water. The results are shown in Table II. It is clear that hydantoin-5-propionic acid is not formed by nonenzymic oxidation of imidazolonepropionic acid.

Reaction of "264 mμ product" with functional reagents and attempts at the isolation of the product: Various attempts to form derivatives of the carbonyl group in the

TABLE II

FAILURE OF NONENZYMIC CONVERSION OF IMIDAZOLONE PROPIONIC ACID TO HYDANTOIN PROPIONIC ACID

Per cent total activity recovered in hydantoin propionic acid in Expt. 1 on basis of specific activity after six recrystallizations = 0.32 %. Recovery in Expt. 2 on basis of specific activity after five crystallizations = 0.40 %.

Expt. No.	*No. of crystallizations*	*Specific activity**	*Total activity in hydantoin propionic acid after correction for losses (counts/min)*
1	3	24.3	4860
	5	7	1400
	6	5	1000
2	3	36	7200
	4	7.5	1500
	5	6.2	1240

* Specific activity is expressed as counts/min/mg after correction for background.

"264 mμ product" (imidazolonepropionic acid) by addition of CN^-, hydroxylamine, semicarbazide or other reagents were without success. The possibility of trapping the lactim form of imidazolonepropionic acid with borate ions in analogy with the experiments of KNOX AND PITT[25] also was of no avail. No change in the spectral characteristics of the "264 mμ product" was noticed by addition of borate ions or by carrying out the urocanase reaction in borate buffer. Since previous experiments indicated that at an acid pH the 234 mμ absorption maximum remained fairly constant for periods of up to 76 h under anaerobic conditions, a large scale incubation using 20 μmoles of urocanic acid was carried out and the reaction terminated with glacial acetic acid.

After removal of the protein, the solution was lyophilized. Only a red material contaminated with inorganic salts was recovered, indicating that the product underwent decomposition.

Nonenzymic degradation of "264 mμ product": FEINBERG AND GREENBERG[6] have reported tentative evidence for the formation of formylisoglutamine from the product of urocanase reaction. Other investigators[26-28] have reported the formation of formylisoglutamine during the enzymic degradation of urocanic acid. The present experiments confirm and extend these observations.

In a representative experiment 200 μmoles of potassium phosphate buffer, pH 7.2, 0.294 μmoles of urocanic acid (27,400 counts/min) were incubated with 220 units of urocanase of specific activity 235 in a total volume of 3 ml. The reaction was terminated after 12 min by addition of 0.4 ml of 10 % perchloric acid and the denatured protein removed by centrifugation. The supernatant fluid was neutralized to pH 7 with 1 *N* KOH and the volume made up to 6 ml. Aliquots were taken for determination of radioactivity. 5 ml of the above solution was applied to a [illegible]owex 1-acetate 30 × 1 cm column and washed with 25 ml of water. The gradient elution device described previously was attached to the column and 3 ml fractions at the rate of 0.3 ml/min were collected. The elution pattern is shown in Fig. 4. The radioactive fractions were individually lyophilized. Fraction C, which contained 53 % of the radioactivity, migrated on paper chromatograms in several solvent systems[4,5] as authentic formyl-L-isoglutamine. Other fractions were not identified. None of the fractions contained formiminoglutamic acid as tested enzymically. In another experiment when formiminoglutamic acid was added as carrier and subjected to ion exchange chromatography in the above manner, most of the formiminoglutamic acid was found in the nonradioactive zones.

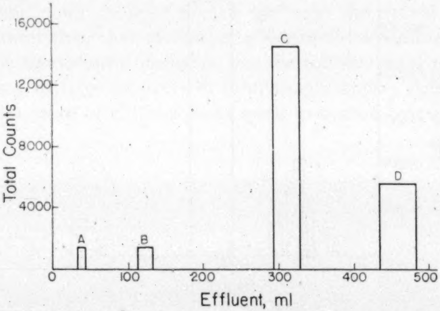

Fig. 4. Chromatographic separation of products of urocanase reaction on Dowex 1-acetate (30 × 1 cm) column.

Isolation of N-formyl-L-isoglutamine

Since sufficient amount of authentic material was not available to perform an isotope dilution experiment on fraction C, a large scale incubation was carried out as described below to secure sufficient N-formyl-L-isoglutamine to establish its identity unequivocally.

1 g of nonradioactive urocanic acid (5740 μmoles) and 15 μmoles of radioactive urocanic acid ($8.7 \cdot 10^6$ counts/min) were dissolved in water with aid of 7 ml of 1 *N* KOH. The pH was adjusted to 7.2 and the volume made up to 200 ml. 90 ml of 0.1 *M* potassium phosphate buffer containing $6.7 \cdot 10^4$ units of urocanase of specific activity 93 were added to the urocanic acid solution and incubated at room temperature for 10 h. The reaction mixture progressively turned yellow during incubation and 90 % of the urocanate disappeared. The reaction was terminated by addition of 5 ml of 60 % perchloric acid and the denatured protein removed by centrifugation at 3°. The supernatant fluid showed absorption maxima at 234 mμ and 260 mμ in the u.v. region and at 365 mμ in the visible region. The pH was cautiously adjusted to 7.2 with 20 % KOH. The spectrum now showed a single u.v. absorption maximum at 266 mμ, which disappeared rapidly with time and an absorption maximum at 398 mμ in the visible region, which did not change over a period of 12 h. The bulk of the solution, which was distinctly yellow, was left overnight at 3° in the refrigerator. The supernatant liquid was decanted from the $KClO_4$ crystals and concentrated to a volume of 35 ml in a rotary evaporator *in vacuo* at 40°.

Samples were taken for determination of radioactivity and the rest of the material was put directly on a Dowex 1-acetate, 33 × 2.5 cm column and washed with 50 ml of water. The radioactive components were eluted from the column as described previously. 8-ml fractions at the rate of 0.8 ml/min were collected. The elution pattern is shown in Fig. 5. The yellow pigment in solution, which turned red during the concentration of the solution, was adsorbed as a band at the surface of the column. Increasing the concentration of the eluting acid gradually removes traces of the red pigment contaminating some of the fractions.

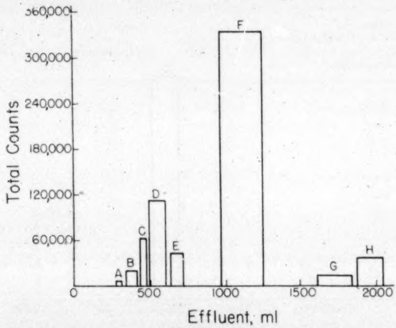

Fig. 5. Chromatographic separation of products of urocanase reaction (large scale incubation) on Dowex 1-acetate column (33 × 2.5 cm)—isolation of N-formyl-L-isoglutamine.

The radioactive fractions were examined on paper for u.v. absorbing material, diazo reagent-reacting substances, ninhydrin-reacting material and for N-substituted amino acids. The results are shown in Table III. The respective fractions were pooled and evaporated at 40° *in vacuo* in a rotary evaporator and 3 to 5 ml of absolute ethanol was added to all the fractions. Fraction "D", which contained approximately 13.4% of the total isotope added to the column, was crystallized from water and identified as unchanged urocanic acid by its u.v. absorption, color tests, chromatographic behavior and infrared spectra. Fraction "F", which contained approximately

TABLE III

RESULTS OF COLOR TESTS AND RADIOACTIVITY DETERMINATION ON VARIOUS FRACTIONS OBTAINED DURING ION-EXCHANGE COLUMN CHROMATOGRAPHY OF PRODUCTS OF UROCANASE REACTION

Explanation of signs: —, negative; ±, doubtful; +++, intense reaction.

Fraction	*Diazo reagent*	*U.v. absorption*	*Ninhydrin reaction*	*Starch–iodide reaction*	*Per cent of total radioactivity*[*]
A	—	—	—	—	0.6
B	—	—	—	—	2.3
C	—	±	—	—	7.6
D	+++	+++	—	—	13.4
E	—	—	±	—	5.1
F	—	—	—	+++	40.8
G	—	±	—	—	1.3
H	—	—	—	—	3.4
Sum of total activity recovered in all individual fractions					74.5

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[*] Approximately 9% of the radioactivity was distributed in various fractions in between the individual peak fractions. The rest of the radioactivity (16%) was held as a pigment on the surface of the column.

41 % of the radioactivity on the column, (or 67 % of radioactivity put on the column after correcting for unchanged urocanic acid), crystallized from ethanol yielding 150 mg of rosette crystals. The compound had a melting point of 133° and the mixed melting point with authentic N-formyl-L-isoglutamine was undepressed. The infrared spectrum of the unknown and authentic samples were identical in every respect (Fig. 6). However, when the sample was crystallized twice from boiling absolute ethanol, the melting point dropped to 128–130° and the optical rotation of a 2 % solution was 6° lower than that of synthetic N-formyl-L-isoglutamine[2]. The infrared spectrum was not significantly changed, but the solution used for optical rotation showed, in addition to large amounts of formylisoglutamine, significant amounts of isoglutamine and a trace of glutamic acid when examined by paper electrophoresis at pH 3.5.

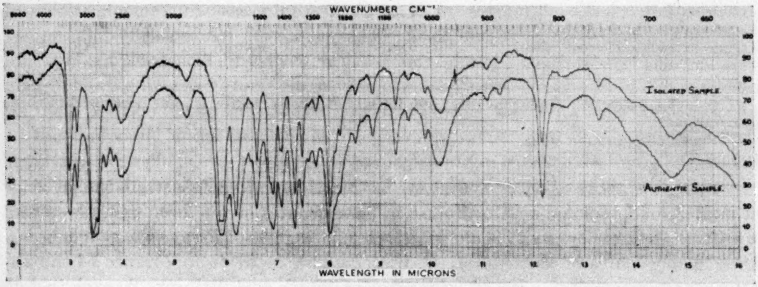

Fig. 6. Infrared spectrum of authentic and isolated samples of N-formyl-L-isoglutamine in Nujol mull. Beckman IR-5 infrared spectrophotometer was used. The peaks at wave lengths of 3.45, 3.52, 6.9, 7.35 represents the absorption of Nujol.

Since none of the original column fractions contained ninhydrin-reacting substances but contained significant amounts of N-substituted amino acids, it can be concluded that N-formyl-L-isoglutamine is the principal nonenzymic degradation product of imidazolonepropionic acid.

Imidazolonepropionic acid ⟶ formiminoglutamic acid

Because of the apparent instability of imidazolonepropionic acid and the failure of attempts to synthesize this compound, we had to resort to a multienzyme system to demonstrate the overall conversion of urocanic acid to formiminoglutamic acid. A typical experiment is described below.

The reaction mixture contained 200 μmoles of potassium phosphate buffer, pH 7.2, 0.2 μmoles of urocanic acid, 260 units of urocanase of specific activity 406 in a total volume of 1 ml. Exactly after 10 min incubation, an excess of formiminoglutamic transferase, and a source of imidazolonepropionic acid hydrolase (crude extracts of various tissues) were added. Finally 0.1 ml of folate·H_4 solution in 1 *M* potassium phosphate buffer, pH 7.2, containing 0.4 mg of folate·H_4 and 15 μmoles of mercaptoethanol were added. The final volume was 1.6 ml. The reaction mixture was incubated for a further period of 30 min at room temperature. The reaction was terminated by addition of 0.4 ml of 10 % perchloric acid and the contents heated for 1 min in a boiling water bath to convert the formimino-FAH$_4$ to 5,10-methenylfolate·H$_4$ and cooled in ice. It was centrifuged to remove denatured protein and KClO$_4$. The O.D. of the clear supernatant liquid was measured at 350 mμ in 1.5 ml cuvettes. Various combinations of the enzymic reaction mixture components were run as blanks to correct for nonenzymic and other side reactions. The results are shown in Table IV.

TABLE IV

ENZYMIC CONVERSION OF IMIDAZOLONEPROPIONIC ACID TO FORMIMINOGLUTAMIC ACID

Experimental details described in text.

Reaction components	*Net O.D. at 350 mμ*	*μmoles of folate·H$_4$ 5,10-methenyl-FA·H$_4$ formed**	*Overall conversion of urocanic acid to formiminoglutamic acid (per cent)*
Complete system	0.77	0.0616	30.8
–crude extract	0.10	0.0080	4.0
–crude extract and FIGLU** transferase	0	0	0
–crude extract and urocanase	0.04	0.0032	1.6
–urocanic acid	0	0	0

* A molar extinction coefficient of 25,000 for 5,10-methenyl folate·H$_4$ has been used in these calculations.

** Formiminoglutamic acid.

Using the above assay procedure we have been able to demonstrate the presence of imidazolonepropionic acid hydrolase in homogenates of rat, beef, calf, sheep and hog livers. A trace of activity could be detected in extracts of *Pseudomonas fluorescens*. Liver homogenates of various species also caused a rapid disappearance of the absorption maximum at 264 mμ of imidazolonepropionic acid. Preliminary experiments indicate that the cleavage of the imidazolone ring does not appear to require divalent metal ions, since supplementation of the above multi-enzyme system with Mg^{++}, Mn^{++}, or Ca^{++} ions did not increase the overall yield of formiminoglutamic acid. Further, the presence of EDTA in the above assay system did not decrease the overall yield. Further studies on the enzyme system which converts imidazolone propionic acid to formiminoglutamic acid are in progress.

DISCUSSION

Previous work by FEINBERG AND GREENBERG[6], has indicated that the enzymic conversion of urocanic acid to formiminoglutamic acid proceeds through an intermediate having an absorption maximum at 264 mμ. These observations have now been confirmed and extended by isotopic experiments with highly purified beef liver urocanase. The enzymic degradation of urocanic acid by the purified enzyme yields a product whose spectral characteristics, stability, lability to oxygen, structure of nonenzymic degradation products (N-formyl-L-isoglutamine) and enzymic degradation product (N-formimino-L-glutamic acid) are strongly indicative of a substituted imidazolone. Evidence that no formiminoglutamic acid was formed in the urocanase reaction was obtained by isotope trapping experiments and also by the failure of the urocanase reaction to couple with the formiminoglutamic transferase system of TABOR AND WYNGARDEN[13]. However, when the same system was supplemented with crude extracts of liver of various species and tetrahydrofolic acid, the overall conversion of urocanic acid to formiminoglutamic acid could be demonstrated. These experiments also demonstrate the existence of the enzyme imidazolonepropionic acid hydrolase, which catalyzes the formation of formiminoglutamic acid from imidazolonepropionic acid.

Highly purified fractions of beef liver urocanase (specific activity of 400 or more) show, in addition to the usual 280 mμ absorption maximum, an inflexion at 290 mμ and indications of another inflexion in the 400–425 mμ region. The cause of these anomalous spectra is not known at the present time. Robinson[29] has also reported similar observations with purified urocanase from chicken liver.

Evidence that the product of urocanase reaction is the suspected 4(5)-imidazolone-5(4)-propionic acid is based primarily on the striking similarity of its spectral characteristics at acid, alkaline and neutral pH to synthetic 4(5)-imidazolone, and 4(5)-imidazolone-5(4)-acetic acid. The last two compounds have been shown by Freter, Rabinowitz and Witkop[30] and Kny and Witkop[31] to be degraded to formiminoglycine and formimino aspartic acid by extracts of *Clostridium cylindrosporum* and *Pseudomonas fluorescens* respectively. Synthetic 2-methyl-5(4)-imidazolone-4(5)-propionic acid ethyl ester, which differs from 4(5)-imidazolone-5(4)-propionic acid in having an ester group on the propionic acid side chain and a methyl group on the 2 position of the imidazolone ring, shows very similar spectral characteristics to the compound under investigation*. However, these spectral characteristics are reversible with change in pH, in contrast to that of 4(5)-imidazolone-5(4)-propionic acid, which is presumably due to the stabilization of the imidazolone ring by substitution at carbon atom 2.

The instability of imidazolones unsubstituted in position 2 is well known[32]. The compound under investigation was labile to base or oxygen in analogy with the imidazolones (unsubstituted in position 2) mentioned above. The formation of colored products during attempted isolation of the enzymic reaction product is probably due to the formation of dimers, a property characteristic of imidazolones[32,33]. Isotopic experiments in the present study have clearly indicated that N-formyl-L-isoglutamine is the principal nonenzymic hydrolytic degradation product of 4(5)-imidazolone-5(4)-propionic acid and that isoglutamine is formed by decomposition of N-formyl-L-isoglutamine during the isolation process. The highly reactive nature of imidazolonepropionic acid may be of some significance in the formation of hydantoin-5-propionic acid, since this reaction involves the oxidation of carbon 2 of the imidazolone ring. It is interesting to note that the reverse reaction, namely, the reduction of hydantoins to imidazolones has been demonstrated by chemical methods[34]. Hydantoin-propionic acid is not formed nonenzymically from imidazolone propionic acid by oxidation with dichlorophenolindophenol. It is probably formed enzymically from imidazolonepropionic acid, although such a reaction has not been demonstrated so far in purified enzyme systems. Hydantoin acrylic acid[35] has been implicated as an enzymic oxidation product of urocanic acid in *Pseudomonas aeroginosa* and in rabbit liver[36].

ADDENDUM

After this paper was written the report of Brown and Kies appeared (*J. Biol. Chem.*, 234 (1959) 3182, 3188) in which the partial purification of 4(5)-imidazolone 5(4)-

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* An alcoholic solution of 2-methyl-4-imidazolonepropionic acid ethyl ester had an absorption maximum at 230 mμ which was shifted to 256 mμ by addition of NaOH. The original 230 mμ maximum reappeared when acidified (D. R. Rao and D. M. Greenberg, unpublished results).

propionic acid on Dowex-50 ion exchange resin was described. They have made identical observations regarding its stability and also demonstrated its enzymic conversion to L-hydantoin 5-propionic acid.

ACKNOWLEDGEMENT

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HYDROXYLAMINE REDUCTASE OF A HALOTOLERANT MICROCOCCUS*

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SUMMARY

A hydroxylamine reductase of hemoprotein nature was prepared from a strain of halotolerant Micrococcus. The hydroxylamine reductase was shown to contain iron as its active center. Manganese ions at 0.5 m*M* concentration activate the reaction about 3 to 4 times when the activity is measured by the reduced methylene blue method; these ions possibly participate in the formation of an hydroxylamine reductase–hydroxylamine complex probably with a simultaneous valency change. The reduced form of this hemoprotein is oxidized by both oxygen and hydroxylamine, the reactions being similarly activated by manganese ions. Carbon monoxide combines with the reduced hemoprotein photo-reversibly. Pyridine hemochromogen from this enzyme has the same absorption maxima as that from mammalian cytochrome *c*. This autoxidizable and carbon monoxide-binding cytochrome *c* (its reduced α-peak is at 554 mμ) which can be obtained from the halotolerant Micrococcus is shown to be hydroxylamine reductase. A possible relationship between the chemical structure and enzyme activity was discussed.

INTRODUCTION

Hydroxylamine reductase (HdR) which catalyzes the stoichiometric reduction of hydroxylamine to ammonia was first isolated in the cell-free state from *Bacillus pumilus var.* as one of enzymes which catalyzed the "successive reduction process" from nitrate to ammonia via hydroxylamine[1]. Several papers on its chief characteristics as well as on the attempts to purify the enzyme have been already published using several strains of halotolerant bacteria[2–4].

Since 1954, pyridine nucleotide-HdR from *Neurospora crassa*[5], soy bean leaves[6], *Azotobacter agile*[7] has been reported. This group of HdR's is considered to have a physiological function in the assimilation of nitrate.

It has been noticed that successive reduction by a halotolerant Micrococcus (Strain 203) is not always reproducible; the Micrococcus grown under the present

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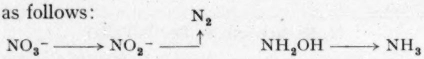
The following abbreviations are used: hydroxylamine reductase, HdR; reduced diphosphopyridine nucleotide, DPNH; cytochrome, cyt.; reduced methylene blue, MbH_2; tris (hydroxymethyl) aminomethane, Tris; methylviologen. MV; reduced MV, MVH.

* Part of this work was read at The International Symposium of Enzyme Chemistry, Tokyo and Kyoto, 1957 (ref. 1).

conditions sometimes does not accumulate ammonia, but instead produces appreciable amounts of nitrogen gas, in either Thunberg tubes or Warburg manometers when formate or MbH_2 is used as the electron donor[8]. The Micrococcus, even when strongly denitrifying, still possesses an active HdR system. Similar observations have also been made with typical denitrifyers such as *Micrococcus denitrificans* and *Pseudomonas denitrificans*[9]. Thus the inorganic nitrogen metabolism in these denitrifying organisms may be expressed as follows:



$$NO_3^- \longrightarrow NO_2^- \longrightarrow \overset{N_2}{\uparrow} \qquad NH_2OH \longrightarrow NH_3$$

It seems, therefore, probable that "HdR" may have a physiological function other than the mere reduction of hydroxylamine[*].

In this paper, further purification and properties of the terminal HdR from the same Micrococcus will be reported. Furthermore its relation to the soluble, autoxidizable and CO-binding cytochrome from the same microbe[11] will also be discussed.

MATERIALS AND METHODS

Microorganism

A halotolerant Micrococcus, Strain 203, was grown aerobically in a 100 l stainless steel tank at 35° for 24 h using the following medium: 700 g of peptone, 700 g of meat extract, 7 kg of NaCl, 700 g of KNO_3, and 14 g of $MgSO_4 \cdot 7H_2O$, in 70 l of tap water, pH 7.2.

Cell-free extract

Grown cells were separated from the medium by a Sharples Supercentrifuge and washed once with 10 % NaCl. The washed cells were added to distilled water where they formed a thick suspension and were lyophilized. From 70 l of medium 120 g of dry cell powder was obtained. The lyophilized bacterial powder was stable for two months at 5° in a vacuum desiccator. To prepare a crude extract, 7–8 g of the powder per 100 ml was suspended in a 10 m*M* phosphate buffer, pH 6.8. The suspension was dialyzed overnight in the cold against a large volume of the same buffer. The dialyzed suspension was centrifuged at $18{,}000 \times g$ for 20 min at 0°. The residue was dissolved in a small amount of the same buffer and again the suspension was centrifuged at $18{,}000 \times g$ for 20 min. These supernatant solutions were combined to give a cell-free dark-brown fluid which contained more than 85 % of the HdR activity in the original suspension. This was used as the crude extract for further purification.

Calcium phosphate gel

A calcium phosphate gel was made according to the method of KEILIN *et al.*[12] with the following modification. The gel was washed eight times with distilled water until the washings were at pH 8. The gel was adjusted to contain 30 to 40 mg/ml. of dry gel. Fresh gel was used without aging and it was stable for at least one month.

Ion exchange resin

An anion-exchange resin, Dowex 2 (cross-linkage 8–10 %), was treated according to the method of BOMAN *et al.*[13].

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[*] Recently it was found that the homogeneously purified HdR from the micrococcus possibly functions as a soluble activator for the particulate nitrite reductase of the same bacterium[10].

Purification procedures

All procedures were carried out below 4° unless otherwise stated.

Acetone fractionation. 5 *M* sodium chloride and 0.2 *M* phosphate buffer, pH 6.8 were added to the crude extract (Preparation 1) to give final concentrations of 0.33 *M* and 0.02 *M*, respectively. Purified acetone which had previously been cooled in a mixture of ice and NaCl at —15° was added carefully to the extract to 40% (v/v). After standing for 15 min at —10°, the precipitate was removed by centrifugation for 10 min at 3000 × *g*. Sufficient acetone was added to the supernatant to give a 52% (v/v) solution of acetone. After 15 min at —10°, the red precipitate formed was collected by centrifugation. It was dissolved in a small volume of distilled water and lyophilized (Preparation 2).

Calcium phosphate gel treatment. 1 g of Prep. 2 was dissolved in 150 ml of 0.01 *M* acetate buffer, pH 5.5, to which about 1 g (dry weight) of calcium phosphate gel was added with stirring. Before carrying out a full-scale experiment, a trial was conducted on a small scale to determine the most favorable conditions. After 10 min the gel with the absorbed HdR was collected by centrifugation, washed twice with the original volume of 33 m*M* phosphate buffer, pH 6.8, and finally eluted twice with 0.1 *M* phosphate buffer, pH 6.8. This eluate was termed Preparation 3.

Ion exchange resin chromatography: Prep. 3 which contained a high concentration of phosphate, was dialyzed against a large volume of distilled water for several hours with mechanical stirring. Then the dialyzed and phosphate-free preparation was passed through a column of the chloride-form of Dowex 2, which had been buffered with 0.02 *M* Tris–HCl buffer, pH 7.3 according to BOMAN *et al.*[13]. Elution was carried out successively with 50 ml of 0.02 *M*, 50 ml of 0.3 *M*, 40 ml of 1.0 *M* Tris–HCl buffer, pH 7.3 and finally 100 ml of 1 *N* HCl. After the dead volume of the column had passed through the column, a 5 ml-fraction was collected in a small test tube by an automatic fraction collector. Three major protein peaks were observed and the HdR activity was found in the last peak which had been eluted by the 1.0 *M* Tris–HCl buffer. This fraction was called Preparation 4. Results of the purification are summarized in Table I.

TABLE I

SUMMARY OF PURIFICATION OF MICROCOCCUS HYDROXYLAMINE REDUCTASE

Preparation No.		Total activity	Specific activity	Per cent recovery
1	Crude extract	1020	4.1	100
2	Acetone fraction (40–52%)	560	17.0	55
3	Calcium phosphate gel eluate	400	130	40
4	Dowex 2 eluate	360	870	36

Electrophoretic and ultracentrifugal analyses

The purity of Prep. 4 was examined with a Hitachi electrophoretic apparatus and a Spinco Model E analytical ultracentrifuge. At least one colorless contaminating component was found in the final preparation.

Measurement of HdR activity

Reduced methylene blue method. HdR activity was measured anaerobically using MbH_2 as an electron donor as described by SATO *et al.*[14] in a final volume of 2.5 ml of reaction mixture containing the following components*: 0.05 M phosphate buffer, pH 6.8; enzyme preparation; 7.5 μmoles MbH_2 (in main compartment of Thunberg tube); 6.25 μmoles hydroxylamine sulfate (in side compartment). After a given time at 37°, the decrease of the hydroxylamine was determined by the method of CSÁKY[16]. In the case of highly purified preparations (Prep. 3 or 4), 1.25 μmoles $MnCl_2$ was added as a specific activator to the main compartment.

Spectrophotometric method. The activity of Preps. 3 and 4 could also be measured spectrophotometrically. The enzyme preparation in 2.9 ml of 50 mM phosphate buffer, pH 6.8, was reduced by the addition of 0.1 ml of fresh 0.02 M sodium dithionite solution in the main compartment of a Thunberg tube-type cuvette and 0.1 ml of 10 mM hydroxylamine sulfate solution was placed in the side compartment. After the evacuation, the reaction could be observed to begin at room temperature by the transfer of the substrate from the side to the main compartment, followed by a change in the optical density at 420 mμ, which was later shown to be caused by a Soret band of the reduced HdR. An Hitachi electrospectrophotometer model ERB-U was employed throughout this work.

Definition of the enzyme units and specific activities

In the MbH_2 method, one unit of HdR was taken to represent the amount of enzyme required to reduce 1.0 μmole of hydroxylamine per hour. In the spectrophotometric method, on the other hand, one unit of HdR was expressed as the amount of enzyme which brought about a drop of 0.001 in the optical density at 420 mμ per min. The specific activities of these were expressed as units of activity per mg of Kjeldahl nitrogen in the enzyme preparation.

Reaction with oxygen

The activity was measured spectrophotometrically in the same way as has been described for the spectrophotometric method except that the gas phase was air without hydroxylamine.

Detection of the valency change of manganese ions during the enzymic reaction

In order to detect the possible occurrence of Mn^{3+} produced from Mn^{2+} during NH_2OH reduction, KAHANE's formaldoxime–Mn^{3+} complex method[17] utilizing the wine red color of the formaldoxime–Mn^{3+} complex was applied. In this case, the composition of the standard reaction mixture was modified by replacing MbH_2 and phosphate buffer, pH 6.8 with 2 μmoles MVH (reduced with Mg powder) and the same buffer, pH 8.0 in a total volume of 2.5 ml, respectively. In addition, 0.2 ml of 10-times diluted formaldoxime reagent (a mixture of hydroxylamine and formaldehyde) and 0.3 ml of 0.07 M NaOH solution were placed in the side tube together with the hydroxylamine solution added as the substrate. The Thunberg tube should be evacuated as completely as possible, since any remaining oxygen could possibly oxidize Mn^{2+} to Mn^{3+} under these conditions. After the violet color of the MVH was removed by the hydroxylamine reduction, the occurrence of a pink color complex was observed. Under the above conditions, the MVH was shown to donate enzymically its electron to hydroxylamine and the formaldehyde was not found to affect the HdR activity. The Thunberg tube-type cuvette was employed to determine the Mn^{3+} produced and the wine red color developed was measured by its optical density at 480 mμ.

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* Several halophilic or halotolerant enzymes the optimum salt concentrations of which are unusually higher than those of ordinary enzymes have been reported from this organism[15]. HdR, however, is neither halophilic nor halotolerant and no extra salt was added to the reaction mixture when the activity was measured by this method. However, this is not the case in the spectrophotometric method.

Absorption spectra

To determine the adsorption spectra of the reduced form, the enzyme was reduced anaerobically by a small amount of sodium dithionite. When the different spectra were obtained, the differences in the optical densities were calculated (reduced minus oxidized) and plotted against the wave-length.

Protein estimation

Protein was determined colorimetrically essentially by the method of LOWRY *et al.*[18].

Chemical analysis of metals

Iron, manganese and molybdenum were determined by the methods described by SANDEL[19], KIYOTA *et al.*[20] and SANDEL[21], respectively.

RESULTS

Effect and modes of action of manganese ions and Michaelis constant on substrate

It was often observed that dialysis of Prep. 3 brought about a marked decrease in the enzymic activity. Various metal ions were added to the dialyzed enzyme preparations and it was found that only $MnCl_2$ at a final concentration of 0.5 m*M* restored the activity (Table II).

TABLE II

ACTIVATION OF DIALYZED MICROCOCCUS HYDROXYLAMINE REDUCTASE BY METAL IONS

Prep. 3 was used as the enzyme preparation.

Preparation		*Specific activity*	*Per cent activation**
Non-dialysed preparation		117	
Dialyzed against distilled water for 15 h at 4°		45	
After preincubation for 30 min at 4° with 0.05 *M* phosphate buffer pH 6.8 and 0.5 m*M*** of:	$MnCl_2$	140	+210
	$MgSO_4$	33	— 27
	Na_2MoO_4	45	0
	MoO_3	50	+ 11
	$FeSO_4$	40	— 11
	$FeCl_3$	48	+ 7
	Na_2WO_4	45	0
	$NiCl_2$	41	— 9
	$CoCl_2$	43	— 4
	$ZnCl_2$	0	—100

* Percent relative to dialyzed preparation.

** The same level of each metal ion concentration was also maintained in the reaction mixture of the standard assay using reduced methylene blue.

The fact that the reproducible value of Michaelis constant (K_m) had not been obtained from the LINEWEAVER–BURK's reciprocal plot for the HdR–hydroxylamine complex led the authors to examine the kinetic relationship between the substrate and $MnCl_2$ concentration.

Curve A in Fig. 1 showing the LINEWEAVER–BURK's plot obtained from the substrate saturation data in the presence of 0.2 mM $MnCl_2$ in the MbH_2 method does not give a clear straight line, especially in the ranges of both the higher and the lower substrate concentrations. This suggests that the $MnCl_2$ concentration required for the maximal activity may vary with the substrate concentration. In fact, a $MnCl_2$ concentration approximately one-fifth of that of hydroxylamine was found to be optimal for the activity and using a constant $MnCl_2/NH_2OH$ molar ratio of 1/5 a smooth straight line (B in Fig. 1) was obtained indicating a definite K_m value of $8.1 \cdot 10^{-4}$ M. These results show that Mn ion as a specific activator can profoundly affect the substrate saturation curve suggesting the possible role of the Mn ions in the formation of the HdR–hydroxylamine complex during the hydroxylamine reduction.

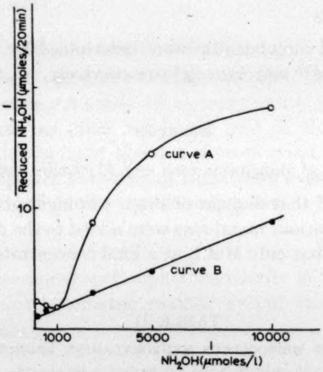

Fig. 1. The effect of NH_2OH and Mn ion concentrations on the reaction rate. Standard assay conditions using reduced methylene blue: curve A, in the presence of 0.2 mM $MnCl_2$; curve B, in the presence of $MnCl_2$ of concentration one-fifth that of NH_2OH.

However, this possibility does not always rule out that in which the Mn ions play an active role in the electron transfer undergoing alternate oxido-reduction. Therefore, the reaction mixture was examined for Mn^{3+} by the formaldoxime method[17]. The violet color of the MVH gradually changed to reddish violet and after the disappearance of the violet color of the MVH the development of clear wine red color could always be easily observed. In a boiled enzyme system, only a very pale orange color could be observed immediately after the decoloration of MVH by gentle shaking in the open air, since no enzymic disappearance of MVH occurred. Owing to the chemical autoxidation of Mn^{2+}, however, this was soon followed by a change to a deep wine red color. When Mn^{2+} was omitted from the complete system, the observed color of the complex after the enzymic decoloration of MVH was very pale orange. Omission of the reduction or MV also gave a pale color presumably due to the slight formation of MVH by formaldehyde. When formaldehyde and NaOH solution were separated from the other components during the incubation and when they were added immediately after the MVH was exhausted, no wine red color could be observed.

These qualitative observations seem to indicate a possible valency change between Mn^{2+} and Mn^{3+} even though the pH condition (8.0) was optimal only for development of the wine-red complex and not for HdR activity. The amount of Mn^{3+} captured as the Mn^{3+}–formaldoxime complex was roughly estimated to be several % of the $MnCl_2$ initially added.

Metal analyses

HdR has long been considered to be one of heavy metal enzymes, since typical heavy metal reagents such as KCN, NaN_3, CO, α,α'-bipyridyl, 8-OH-quinoline and *o*-phenanthroline exert marked inhibitory effects on HdR activity. Therefore, using the partially purified enzyme preparations (Prep. 2 and 3), an attempt was made to analyze the metal constituents by chemical means. Of the three metals determined, only iron was found in dialyzed Prep. 3 in significant quantities (Table III).

TABLE III

METAL CONTENTS IN MICROCOCCUS HYDROXYLAMINE REDUCTASE PREPARATION

Enzyme preparation	*Metal contents (μg/mg N)*					
	Fe		*Mn*		*Mo*	
	Prep. 2	*Prep. 3*	*Prep. 2*	*Prep. 3*	*Prep. 2*	*Prep. 3*
Before dialysis	2.24	2.03	0.42	2.10	0.00	0.00
After dialysis*	1.05	2.03	0.14	0.00	—	—

* Dialysis was carried out at 4° for 24 h against deionized water.

This observation confirms the results of the inhibition experiments[2,3] which suggested the presence of iron in HdR. Manganese, on the contrary, which was present in undialyzed Prep. 3, completely disappeared on dialysis. This fact is particularly significant, since in highly purified preparations, Mn ions must be added to obtain full activity of HdR (Table II). Molybdenum was not found in any of the preparations.

Absorption spectra and cytochrome nature of HdR

Spectrophotometric analysis of Prep. 4 showed that HdR had the typical absorption spectra of a cytochrome (Fig. 2).

In the oxidized form, two bands were seen at 405 mμ and 635 mμ, while in the reduced form, three bands were found at 420 mμ, 521 mμ, and 554 mμ, two of which had shoulders at 434 mμ and 548 mμ. The data presented in Table IV show that these peaks are different from those of two types of cyt. b_4, types I and II[22] which are present in abundance in this halotolerant bacterium.

In fact, these two types of cyt. b_4 were shown to have no HdR activity by both MbH_2 and spectrophotometric methods*.

The method for preparing free heme described by PAUL which had been successfully applied to mammalian cyt. c[23] was first tried for this pigment. The method employs silver nitrate to break the thioether bonds between the heme and the protein. Only part of the heme was liberated into the ether–acetic acid layer. Therefore, pyridine hemochromogens of Prep. 4, as well as purified cyt. b_4, type I, and beef heart cyt. *c*, were prepared by alkaline treatment in the presence of pyridine in order to determine the heme structure. The absorption maxima of these three hemochromogens at α-, β-, and Soret bands were identical, indicating a common heme structure for the *c*-type cytochrome.

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* In this case, the change of the optical density at 418 mμ (Soret band of reduced cyt. b_4) was observed.

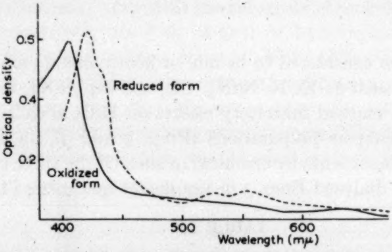


Fig. 2. Absorption spectra of Micrococcus hydroxylamine reductase preparation. Components: hydroxylamine reductase, Prep. 4; phosphate buffer, 0.05 *M*, pH 6.8; total volume, 3.0 ml. Reduced by dithionite.

TABLE IV

ABSORPTION PEAKS OF MICROCOCCUS CYTOCHROMES

Cytochromes	*Absorption peaks (mμ)*	
	Reduced form	*Oxidized form*
Cyt. b_4 type I	554, 548, 521, 418	523, 414
type II	554, 521, 418	523, 414
Hydroxylamine reductase	554, 521, 420	635, 405

Spectrophotometric measurement of HdR activity

As shown in Fig. 3, under the assay conditions of the spectrophotometric method, the optical density of reduced HdR at 420 mμ in Prep. 4 decreased after the addition of hydroxylamine when the reduced spectrum shifted completely to the oxidized one, after which no further decrease in optical density was observed. The presence of Mn ions was again found to stimulate the rate of the reaction.

Reaction of reduced HdR with molecular oxygen

The indication that HdR was a cytochrome led the authors to examine the reaction of HdR with oxygen. It was found that the reduced enzyme reacted with oxygen and that the reaction was again accelerated by Mn ions (Fig. 4). The rate of oxidation of reduced HdR by oxygen is higher than that by hydroxylamine.

There is a linear relationship between the specific activities of HdR measured by the MbH_2 method and of the autoxidizability measured by the spectrophotometric method (Fig. 5), which indicates that HdR can react with two substrates.

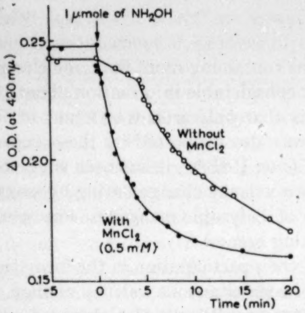


Fig. 3. Oxidation of reduced form of hydroxylamine reductase preparation by NH_2OH and the effect of Mn ions on the oxidation. Standard spectrophotometric assay conditions using Prep. 4.

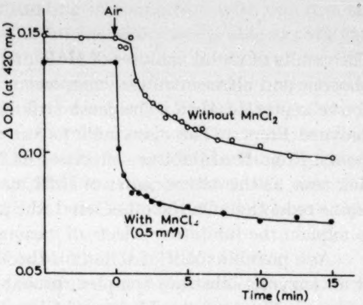


Fig. 4. Oxidation of reduced form of hydroxylamine reductase preparation by oxygen and the effect of Mn ions. Standard spectrophotometric assay conditions using Prep. 4.

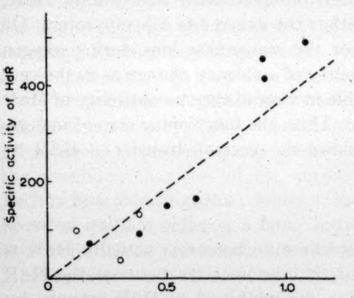


Fig. 5. Relation between hydroxylamine reductase activity and autoxidizability of various preparations: ●, Prep. 4; ○, Prep. 3.

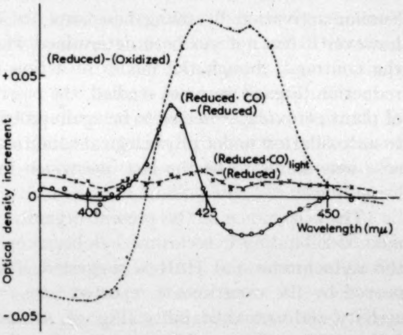


Fig. 6. CO-binding difference spectrum and photodissociation. Standard spectrophotometric assay conditions using Prep. 4. For details see text.

Reaction with carbon monoxide

Using the MbH_2 method, photoreversible inhibition of the HdR activity by CO had been reported previously[3,4]. To confirm this effect of CO on HdR spectrophotometrically, one atm. of CO was introduced into a Thunberg tube-type cuvette which contained reduced HdR.

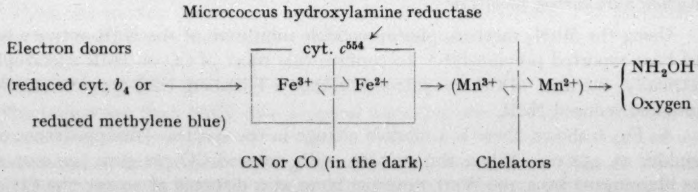
As Fig. 6 shows, there is a marked change in the spectra—disappearance of the shoulder at 434 mμ. When the cuvette, with reduced CO pressure (*ca.* 0.02 atm.) was illuminated by a 100 Watt tungsten lamp at a distance of 30 cm, the CO effect was completely reversed. No photoreversibility was observed with less than 1 atm. of CO. These observations confirm the previous experiments with the MbH_2 method on the inhibitory effects of CO on HdR.

DISCUSSION

The results of metal analyses of HdR preparations containing more than one electrophoretic and ultracentrifugal component, give us considerable information about the active center of HdR. The most striking fact is that only iron was found in the dialyzed Prep. 3. The dissociable nature of Mn was also supported by these experiments. From the inhibitory effects of KCN and CO on HdR[3,4], it has been suggested that iron, as the active center of HdR, undergoes a valency change during hydroxylamine reduction. On the other hand, the presence of dialyzable manganese ions seems to explain the inhibitory effects of various chelating agents[3,4].

As a possible mode of action for the Mn ions, their participation in the formation of an enzyme–substrate complex, probably with a simultaneous valency change, is proposed. The reproducible straight line in Lineweaver-Burk's plot obtained with a constant $MnCl_2/NH_2OH$ molar ratio of 1/5 can be also explained by the assumption that the true substrate for the HdR is not free hydroxylamine but a complex of hydroxylamine and Mn ions. Unlike the case of the complex ion $(MgP_2O_7)^{=}$ supposed to be the true substrate for rat erythrocyte pyrophosphatase[24] however, neither evidence nor any indication of the existence of such a complex has been put forward. Similar activation by manganese ions has been observed with Azotobacter HdR; however it has not yet been determined whether the enzyme is a hemoprotein. On the contrary, though the modes of action for the manganese ions during oxygen reduction (Fig. 4) were not studied, the possibility of a valency change as in the case of plant peroxidase[25] seems to be again probable in view of the susceptibility of Mn^{2+} to autoxidation under physiological conditions. Thus, the functioning step of manganese ions seems to be the last one which follows the electron transfer of HdR by heme irons.

The occurrence in the present organism of a soluble, autoxidizable and carbon monoxide-binding cytochrome has been reported[11] and a possible relation between this cytochrome and HdR is suggested. That the cytochrome is actually HdR is proved by the experiments reported here—firstly, the linearity between the HdR activity and autoxidizability (Fig. 5), secondly, the inhibition of HdR activity by CO[3,4] and its effect on HdR spectra (Fig. 6) and finally the spectroscopic change of the absorption band caused by the addition of hydroxylamine (Fig. 3). Therefore from these results HdR can be represented as cyt. c^{554} (halotolerant Micrococcus, CO-binding). The above findings are summarized schematically in the following scheme:



Some heme proteins such as human hemoglobin[26] and an unidentified cytochrome found in mammalian liver[27] have been reported to react with hydroxylamine in a similar manner to that described above. Observations recently reported by Ishimoto *et al.*[28] and SENEZ *et al.*[29] are essentially interesting in connection with our results. They found that cyt. c_3 produced from *Desulfovibrio desulfuricans* is able to catalyze the reduction of hydroxylamine by MVH. This HdR activity is inhibited by CO; this inhibition was reversed by light and the reduction was remarkably stimulated by Mn ions. The HdR activity of cyt. c_3 could also be confirmed by our MbH_2 method. MINAKAMI *et al.*[30] have also shown that heme-peptides prepared from mammalian cyt. *c* by tryptic digestion possess both HdR activity and CO-binding capacity, although intact cyt. *c* does not. The effect of Mn ions was, however, not observed in this case. These findings seem to suggest that cytochromes of the *c*-type are able to acquire an HdR activity and CO-binding capacity if their structures are modified moderately. It seems, however, quite improbable that the HdR obtained by us is an artifact in view of the mildness of the procedures employed and the consistent amounts recovered in each step of the purification.

In this connection it is noteworthy to mention that cyt. b_4 is completely inert toward hydroxylamine as well as toward oxygen and CO in the reduced form. Recently, *Rhodospirillum rubrum* hemoprotein[31] has been shown to have properties quite similar to that of HdR, although its reduced form lacks a β-band. Recently, these and chromatium cytochromes[32] and CHANCE's "new-CO-binding pigment", *e.g.*, from *Staph. albus*[33] have been tentatively classified by MORTON[34] under the new heading of "cyt. *d*" which is characterized by *c*-heme, autoxidizability and CO-binding capacity. Further extensive studies seem to be still required before those findings can be generalized to give the characteristics of a certain group of hemoproteins.

The presence of CHANCE's "new CO-binding pigment" or "cyt. *o*"[35] which is regarded as a new type of terminal oxidase has been also confirmed in the halotolerant Micrococcus employed in this study[36]. No evidence, however, has been obtained for the possibility that the soluble, autoxidizable and CO-binding cyt. c^{554} which shows HdR activity is a terminal oxidase functioning *in vivo*—on the contrary, almost all of *p*-phenylene diamine oxidizing activity originally present in the lyophilized cells was found in the precipitate after centrifugation (105,000 × *g* × 60 min), while the HdR activity was found exclusively in the supernatant. Furthermore, the abundant presence of a CO-binding cytochrome in the precipitate has been confirmed. These observations seem to rule out the possibility that HdR functions as the predominant terminal oxidase in the Micrococcus.

The final purification and the study of the physiological significance of HdR, as well as the biochemical comparison of HdR with an uncharacterized particulate CO-binding cytochrome found in the same Micrococcus are now under way.

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FACTEURS INTERVENANT DANS LA PHOSPHORYLATION OXYDATIVE: ÉTAT D'OXYDO-RÉDUCTION DES TRANSPORTEURS D'ÉLECTRONS ET pH

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SUMMARY

Factors affecting oxidative phosphorylation: redox condition of electron carriers and pH

1. The effect was studied of the pH and of the redox condition of the electron carriers in the DPN...O_2 chain, on the partial reactions in oxidative phosphorylation that are catalysed by submitochondrial particles.

2. The rate of oxidation of β-hydroxybutyrate, the efficiency of the phosphorylation connected with it, and the rate of the ATP–P_i exchange are characterized by two pH optima, at 6.5 and 7.3. ATPase activity is characterized by three pH optima, at 6.5, 7.3 and 8.5.

3. Partial, nearly complete and complete reduction states of the DPN ... O_2 respiratory chain or parts thereof were obtained by means of the inhibitors amytal, antimycin A, cyanide, or by means of total anaerobiosis.

4. At pH 6.5, nearly complete or complete reduction of the electron carriers situated between DPN and cytochrome *b* or between DPN and cytochrome a_3 increases ATPase activity and reduces the rate of the ATP–P_i exchange, whereas at pH 7.3 both the ATPase activity and the rate of ATP–P_i exchange are diminished.

5. At pH 7.3, a semi-reduced condition of the electron carriers located between DPN and cytochrome *b* stimulates both the ATPase activity and the ATP–P_i exchange.

6. The results obtained suggest that there are two possible mechanisms of oxidative phosphorylation as regards the transfer of electrons from DPNH to cytochrome *b*. These mechanisms are discussed in the light of the experimental results.

INTRODUCTION

L'oxydation d'une molécule de DPNH par des mitochondries s'accompagne de la phosphorylation de 3 molécules d'ADP[1,2], chaque phosphorylation étant probablement associée à un transfert d'électrons entre les transporteurs: DPN et flavoprotéine,

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Abréviations: DPNH et DPN$^+$: diphosphopyridine nucléotide réduit et oxydé; ATP et ADP: adénosine di- et triphosphate; P_i: orthophosphate; Tris: tris(hydroxyméthyl)amineméthane; EDTA: éthylène-diaminetétracétate de sodium; amytal: éthyl-5-isoamyl-5-barbiturate, DNP: 2,4-dinitrophénol.

flavoprotéine et cytochrome *c*, cytochrome *c* et oxygène[3–7]. Les différents schémas proposés actuellement pour expliquer le mécanisme de la phosphorylation oxydative[6, 8–11] peuvent être classés en 3 catégories:

Schéma I:

$$T + P_i \xleftrightarrow{\pm 2e} T \sim P_i \tag{1}$$

$$T \sim P_i + ADP \longleftrightarrow T + ATP \tag{2}$$

Schéma II:

$$T + I \xleftrightarrow{\pm 2e} T \sim I \tag{3}$$

$$T \sim I + P_i \longleftrightarrow T + P \sim I \tag{4}$$

$$P \sim I + ADP \longleftrightarrow I + ATP \tag{5}$$

Schéma III:

$$T + I \xleftrightarrow{\pm 2e} T \sim I \tag{3}$$

$$T \sim I + X \longleftrightarrow T + X \sim I \tag{6}$$

$$X \sim I + P_i + ADP \longleftrightarrow I + X + ATP \tag{7}$$

où T représente un transporteur d'électrons, X un intermédiaire hypothétique, I un inhibiteur qui serait présent dans les mitochondries et dont la combinaison avec T est déplacée par P_i ou par X. On admet que les liaisons entre T et P_i, T et I, P_i et I ou encore entre X et I sont de même niveau énergétique que la liaison pyrophosphate terminale de l'ATP. Les réactions de transfert (1) et (3) présentent chacune deux alternatives aboutissant à un dérivé "riche en énergie" dans lequel T est soit oxydé soit réduit. Ainsi la réaction (1) s'écrira:

$$T_{1\,\text{red}} + T_{2\,\text{ox}} + P_i \longleftrightarrow T_{1\,\text{ox}} \sim P_i + T_{2\,\text{red}} \tag{1a}$$

ou bien

$$T_{1\,\text{red}} + T_{2\,\text{ox}} + P_i \longleftrightarrow T_{1\,\text{ox}} + T_{2\,\text{red}} \sim P_i \tag{1b}$$

Quel que soit le schéma invoqué, il est difficile de préciser si T est sous un état oxydé (alternative a) ou réduit (alternative b) dans les dérivés $T \sim P_i$ et $T \sim I$, les résultats publiés jusqu'à présent[12–15] aboutissant à des conclusions différentes.

On peut tenter de résoudre le problème de la formation des dérivés "riches en énergie" et celui du choix entre les schémas I, II et III par l'étude de deux processus bien individualisés qui font partie de la phosphorylation oxydative: l'hydrolyse de l'ATP et l'échange ATP–P_i en l'absence de tout substrat exogène oxydable. Les processus d'hydrolyse et d'échange sont, en effet, impliqués dans les schémas I, II et III à des niveaux différents: au niveau des réactions (1) et (2) dans le schéma I, des réactions (4) et (5) dans le schéma II, de la réaction (7) dans le schéma III; ils dépendent dans le schéma I d'un transfert d'électrons entre 2 transporteurs T_1 et T_2 et par conséquent de l'état d'oxydo-réduction des deux transporteurs T_1 et T_2; selon le schéma II, ils ne sont liés qu'à l'état d'oxydo-réduction d'un seul transporteur; enfin selon le schéma III ils s'accomplissent sans qu'aucun transporteur n'intervienne et indépendamment de tout changement d'état d'oxydo-réduction.

Dans le cas général du schéma I, les vitesses d'échange ATP–P_i et d'hydrolyse de l'ATP dépendent à la fois de la proportion des formes oxydée et réduite des deux transporteurs T_1 et T_2 et de la constante d'équilibre K de la réaction globale:

$$T_{1\,\text{red}} + T_{2\,\text{ox}} + P_i + ADP \longleftrightarrow T_{1\,\text{ox}} + T_{2\,\text{red}} + ATP$$

Par exemple, si K est voisin de 1, ces vitesses sont maxima lorsque les proportions des formes oxydée et réduite de T_1 et T_2 sont du même ordre de grandeur*. De toutes façons, dans le schéma I, une oxydation complète ou une réduction complète de T_1 et de T_2 doit entraîner un ralentissement des processus d'échange et d'hydrolyse. Dans le cas du schéma II, suivant que T dans T $\sim$ I est sous un état réduit, oxydé ou de type "semi-quinonique", les vitesses d'échange et d'hydrolyse sont maxima pour un état complètement réduit, complètement oxydé ou semi-réduit de la portion de chaîne où T se trouve localisé.

Dans ce mémoire, les variations de la vitesse d'échange ATP–P_i et de la vitesse d'hydrolyse de l'ATP sont étudiées à différents pH en fonction de l'état d'oxydo-réduction des transporteurs de la chaîne respiratoire DPN . . . O_2. Les expériences ont été réalisées avec des particules sub-mitochondriales préparées à partir de foie de rat. Les résultats obtenus conduisent à admettre que le schéma I ou l'alternative "semi-quinonique" du schéma II représentent des mécanismes possibles de phosphorylation oxydative pour un transfert d'électrons du DPNH au cytochrome *b*.

MATÉRIEL ET MÉTHODES

Préparation de fragments de membranes de mitochondries

Les fragments de mitochondries sont préparés par un procédé voisin de celui décrit par DEVLIN ET LEHNINGER[16]. Les mitochondries de foie de rat (rats Wistar adultes de 150 à 200 g) sont d'abord isolées suivant la méthode de SCHNEIDER ET HOGEBOOM[17] et lavées deux fois avec une solution de saccharose 0.25 *M*. On homogénéise alors le sédiment mitochondrial dans une solution refroidie de digitonine purifiée à 1 % (5 ml de solution de digitonine pour un foie). Après 30 min de contact à 2°, la suspension est centrifugée à 35.000 × *g* pendant 15 min (Ultracentrifugeuse Spinco, Rotor 40.2). Le sédiment constitué par des mitochondries non lysées et par de gros débris mitochondriaux est éliminé; le surnageant est centrifugé à 110,000 × *g* pendant 30 min. Les fragments ainsi isolés sont remis en suspension dans de l'eau distillée et utilisés immédiatement pour les expériences. Toutes les opérations sont conduites à une température voisine de 2°. Le rendement en particules sub-mitochondriales obtenues à partir de mitochondries entières a été calculé d'après les teneurs en azote; il varie entre 10 et 15 %.

Produits chimiques et leur purification

Les produits chimiques utilisés sont des produits commerciaux: ATP et ADP (Sigma Chemical Company), Antimycine A (Wisconsin Alumni Research Foundation). La digitonine (Hoffmann La Roche) est purifiée de la façon suivante: 5 g sont dissous dans 24 ml de soude N auxquels on ajoute 16 ml d'EDTA 0.1 *M*. Afin de précipiter la digitonine on ajoute trois volumes d'acétone refroidie à 0°. Il se forme un gel blanchâtre qu'il est aisé de recueillir sur les parois d'un agitateur. On dissout le gel dans 5 ml d'eau et on le précipite à nouveau par de l'acétone. On effectue une troisième fois la même opération et on lave finalement le gel par de l'acétone pure. Après dessication sous vide, on obtient la digitonine sous forme d'une poudre soluble dans l'eau. Le pH de la solution aqueuse est ajusté à 7 par addition d'acide chlorhydrique *N*.

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* Un cas particulier du schéma I est celui où T dans T $\sim$ P_i est de type "semi-quinonique".

Les méta- et pyrophosphates qui accompagnent souvent à l'état de traces l'orthophosphate radioactif ^{32}P sont éliminés par hydrolyse acide suivant la méthode de NIELSEN ET LEHNINGER[3].

L'amytal (Laboratoires Roussel) a été recristallisé deux fois dans de l'éthanol en présence d'EDTA.

Mesure de l'échange ATP–P_i

L'incubation se fait dans de microfioles à fond plat agitées dans un thermostat à 25°. Chaque fiole contient: ATP 0.0075 *M*, orthophosphate 0.0001 *M* à 0.05 *M* marqué par ^{32}P, 1 à 2.10^6 coups/min, EDTA 0.0025 *M* et 100 µl de la suspension de fragments sub-mitochondriaux (50 à 150 µg d'azote). Le volume final est de 400 µl. L'orthophosphate sert de tampon entre pH 6.5 et pH 8.5, l'ATP entre pH 5.5 et 7.5. En dehors de ces zones le pH de l'ATP et celui de l'orthophosphate sont ajustés approximativement et maintenus à la valeur désirée par l'addition de tampons appropriés: tampon acétate–acide acétique pour les pH inférieurs à 5.5, tampon Tris–HCl pour les pH supérieurs à 7.0 (dans les deux cas concentration finale 0.015 *M*). Aucun substrat oxydable exogène n'est ajouté au milieu d'incubation.

La vitesse de l'échange étant constante pendant les 30 premières minutes d'incubation dans nos conditions expérimentales (Tableau II), la durée de l'incubation se limite, en général, à 15 min. Les réactions enzymatiques sont stoppées par addition de 100 µl d'acide trichloracétique à 30 % et les protéines éliminées par centrifugation. L'orthophosphate et l'ATP contenus dans une partie aliquote du surnageant (40 à 80 µl) sont séparés par chromatographie sur papier Whatman No 1 avec une phase éther isopropylique–acide formique[18]. Après révélation, les zones correspondant aux taches d'orthophosphate et de phosphates organiques sont découpées et minéralisées dans 500 µl du mélange: 2 parties d'acide perchlorique à 60 % et 3 parties d'acide sulfurique concentré pur. L'acide phosphorique est extrait de la totalité de l'échantillon minéralisé par de l'isobutanol et dosé colorimétriquement[19,20]. Une partie aliquote de l'extrait isobutanolique est déposée sur une coupelle et la radioactivité mesurée après évaporation. Dans quelques expériences on a mesuré directement la radioactivité des taches.

La vitesse de l'échange ATP–^{32}P est calculée d'après la formule de STEWART[21] lorsque la concentration en orthophosphate varie peu durant l'incubation: cette condition est remplie lorsque la concentration en orthophosphate initialement présente est relativement importante (égale ou supérieure à 0.01 *M*). Dans le cas contraire l'ordre de grandeur de l'échange ATP–P_i est évalué d'après le rapport:

$$\frac{\text{radioactivité spécifique de } P\gamma \text{ de l'ATP}}{\text{radioactivité spécifique de l'orthophosphate}}$$

Mesure de l'ATPase

Le milieu d'incubation type (volume final 400 µl) contient: ATP 0.0075 *M*, EDTA 0.0025 *M* et 100 µl de la suspension de fragments de mitochondries (50 à 150 µg d'azote). Les tampons sont employés dans les mêmes conditions que précédemment. Le phosphate libéré par hydrolyse de l'ATP est dosé après séparation chromatographique sur papier. Dans nos conditions expérimentales la vitesse d'hydrolyse est constante pendant les 30 premières minutes d'incubation (Tableau I).

Mesure de la phosphorylation oxydative

A moins que des conditions spéciales ne soient précisées, le milieu d'incubation type (volume final 400 µl) est le suivant: β-hydroxybutyrate 0.01 *M*, EDTA 0.0025 *M*, orthophosphate 0.025 *M* marqué par ^{32}P, $1 \cdot 10^6$ coups/min, ADP 0.0073 *M*. Les solutions tampons à différents pH sont utilisées comme dans la réaction d'échange. La vitesse d'oxydation du β-hydroxybutyrate est estimée égale à la vitesse de formation de l'acétoacétate[1, 22]. L'acétoacétate formé est dosé suivant la méthode de WALKER[23]. Les phosphates sont séparés par chromatographie sur papier[18] et la quantité d'ATP synthétisé est calculée d'après la radioactivité de la tache correspondant aux phosphates organiques et la radioactivité spécifique de l'orthophosphate.

Autres déterminations

La radioactivité spécifique du dernier phosphate (Pγ) de l'ATP et celle de l'avant-dernier phosphate (Pβ) sont mesurées après hydrolyse de l'ATP à 0° par de l'apyrase selon la méthode de WHITTAM *et al.*[24].

Les nucléotides pyridiniques (DPN$^+$ et DPNH) présents dans les fragments sub-mitochondriaux sont extraits respectivement en milieu acide et en milieu alcalin selon GLOCK ET MCLEAN[25] et dosés au spectrophotomètre Beckman DU à 340 mµ, en présence d'alcool déshydrogénase, d'éthanol ou d'acétaldéhyde[26].

L'azote des préparations enzymatiques est dosé après minéralisation et entraînement de l'ammoniaque dans un appareil de PARNAS ET WAGNER. Cependant, dans plusieurs expériences la quantité de protéines a été évaluée par la méthode du biuret[27] et la valeur trouvée rapportée à son équivalent en azote.

Emploi des inhibiteurs respiratoires dans l'étude de la réaction d'échange ATP–P$_i$, de l'hydrolyse de l'ATP et de la phosphorylation oxydative

On sait que l'amytal[28, 29] et l'antimycine A[30] inhibent les transferts d'électrons respectivement entre DPN et flavoprotéine et au niveau du cytochrome *b**. L'amytal et l'antimycine A sont dissous dans de l'éthanol à 70 %; 4 µl de cette solution alcoolique sont ajoutés au milieu d'incubation (400 µl). Dans les expériences où la concentration finale en amytal dépasse 10^{-3} *M*, le propylène glycol sert de solvant à l'amytal et l'on ajoute 10 µl de la solution au milieu d'incubation. (Il est à noter que le propylène glycol entraîne par lui-même une inhibition partielle — 20 à 30 % — de la réaction d'échange et de la vitesse d'oxydation du β-hydroxybutyrate).

Le cyanure est utilisé, le plus souvent, en présence de β-hydroxybutyrate afin d'assurer une réduction plus complète de la partie de chaîne DPN . . . cytochrome a_3[14]. La suspension de fragments submitochondriaux est d'abord mise en préincubation pendant 2 min à 25° avec du cyanure $8 \cdot 10^{-3}$ *M* et du β-hydroxybutyrate $8 \cdot 10^{-3}$ *M*, puis des portions aliquotes de 100 µl sont prélevées et ajoutées au milieu d'incubation type pour la mesure de l'échange ATP–P$_i$ (volume final 400 µl).

Les conditions d'anaérobiose complète sont réalisées dans de microfioles de WARBURG: un bâtonnet de phosphore blanc est introduit dans le godet central et l'on fait passer pendant 3 min un courant d'azote.

L'effet du cyanure et de l'antimycine A sur l'état d'oxydo-réduction des transporteurs d'électrons dans les fragments sub-mitochondriaux a été déterminé en présence et en absence de β-hydroxybutyrate, dans des conditions analogues à celles utilisées pour la mesure de l'échange ATP–P_i.

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* Si l'on se réfère au schéma de la chaîne respiratoire DPN . . . O$_2$ donné par CHANCE ET WILLIAMS[6].

RESULTATS

Caractéristiques des fragments de membranes de mitochondries en relation avec les réactions de la phosphorylation oxydative

L'hydrolyse de l'ATP et l'échange ATP–P_i catalysés par des particules sub-mitochondriales sont particulièrement sensibles à l'EDTA. L'action de l'EDTA sur l'ATPase a été évaluée en présence et en absence de 2,4-dinitrophénol (Tableau I).

TABLEAU I

EFFET DE L'EDTA SUR L'ATPASE EN PRÉSENCE ET EN ABSENCE DE DNP

Conditions: ATP 0.0075 M pH 7.0, éventuellement DNP $1 \cdot 10^{-4}$ M et EDTA à la concentration indiquée, fragments sub-mitochondriaux 100 μg N appartenant à la préparation I ou II. Volume final 400 μl; température, 25°.

Préparation enzymatique	*Temps d'incubation (min)*	*EDTA × 10^{-4} M*	*P_i libéré*		*Activité ATPase (avec DNP) / Activité ATPase (sans DNP)*
			Avec DNP µmoles	*Sans DNP µmoles*	
I*	15	0	1.00	0.29	3.4
	15	0.25	1.05	0.25	4.2
	15	7.5	1.13	0.23	4.9
	30	7.5	2.14	0.43	5.0
II**	15	0	0.53	0.32	1.7
	15	0.25	0.45	0.23	2.0
	15	2.5	0.40	0.15	2.7
	15	7.5	0.39	0.14	2.8
	30	7.5	0.75	0.30	2.5

* Préparation de fragments sub-mitochondriaux I: la digitonine qui a servi à la lyse des mitochondries a été traitée préalablement par de l'EDTA suivant la méthode indiquée dans MATÉRIEL ET MÉTHODES.

** Préparation de fragments sub-mitochondriaux II: la digitonine a été utilisée sans purification préalable.

Puisque la stimulation de l'hydrolyse de l'ATP par le 2,4-dinitrophénol est un phénomène caractéristique de la phosphorylation oxydative, le rapport

$$R = \frac{\text{Activité de l'ATPase en présence de 2,4-dinitrophénol}}{\text{Activité de l'ATPase en absence de 2,4-dinitrophenol}}$$ peut être considéré comme un indice de l'activité du système plurienzymatique impliqué dans la phosphorylation oxydative. R a été déterminé pour deux types de préparation de fragments sub-mitochondriaux (Tableau I). Les fragments de la préparation I proviennent de mitochondries lysées par de la digitonine qui avait été préalablement traitée par de l'EDTA; en ce qui concerne la préparation II, la digitonine utilisée n'avait pas été traitée par l'EDTA. Le rapport R obtenu avec la préparation I est environ deux fois plus grand que celui obtenu avec la préparation II. En outre, dans le cas des deux préparations, l'addition d'EDTA au milieu d'incubation accroît sensiblement la valeur de R. La vitesse de l'échange ATP–P_i et le rendement de la phosphorylation associée à l'oxydation du β-hydroxybutyrate (Tableau II) sont également augmentés

par l'EDTA. Par conséquent, l'EDTA protège ou maintient le système enzymatique de phosphorylation dans les particules sub-mitochondriales.

TABLEAU II

EFFET DE L'EDTA SUR L'ÉCHANGE ATP–^{32}P ET SUR LA PHOSPHORYLATION D'ADP ASSOCIÉE À L'OXYDATION DU β-HYDROXYBUTYRATE

Conditions: Echange ATP–P_i: ATP 0.0075 M, pH 6.5, tampon phosphate marqué par ^{32}P 0.016 M, pH 6.5. Phosphorylation oxydative: ADP 0.0075 M, pH 6.5, tampon phosphate marqué par ^{32}P 0.014 M, pH 6.5 β-hydroxybutyrate 0.010 M. Dans les deux cas, EDTA à la concentration indiquée, fragments mitochondriaux 80 μg N; température, 25°.

Temps d'incubation (min)	*EDTA M*	*P_i échangé mμmoles*	*ATP synthétisé mμmoles*	*Acétoacétate formé mμmoles*	$\frac{P}{O}$
15	0	105	166	149	1.11
15	$5.0 \cdot 10^{-4}$	124	204	146	1.40
15	$1.0 \cdot 10^{-3}$	137	246	146	1.68
15	$2.5 \cdot 10^{-3}$	139	240	143	1.68
15	$5.0 \cdot 10^{-3}$	141	233	140	1.66
15	$7.5 \cdot 10^{-3}$	132	226	136	1.66
30	$2.5 \cdot 10^{-3}$	264			
60	$2.5 \cdot 10^{-3}$	500			

L'addition de Mg^{++} n'est nécessaire ni à l'échange ATP–P_i, ni à la phosphorylation oxydative, ce qui confirme les résultats de COOPER ET LEHNINGER[11, 22]. Mg^{++} est même inhibiteur à des concentrations relativement faibles: par exemple à pH 6.5 et pour une concentration en Mg^{++} égale à $6 \cdot 10^{-4}$ M, la vitesse d'échange ATP–P_i est diminuée de moitié tandis que la vitesse d'hydrolyse de l'ATP est augmentée de 9 fois. Le fait qu'il existe une quantité notable de Mg^{++} dans les fragments sub-mitochondriaux[31] et que l'EDTA stabilise le Mg^{++} des mitochondries sous une forme liée[32] suggère que la forme liée du Mg^{++} intraparticulaire est fonctionnelle et indispensable aux réactions de la phosphorylation oxydative.

Au cours de l'échange ATP–^{32}P, le dernier phosphate $P\gamma$ de l'ATP se marque beaucoup plus rapidement que l'avant dernier phosphate $P\beta$. Ainsi, après 15 min d'incubation à 25° en présence d'une quantité de fragments de mitochondries correspondant à 100 μg d'azote, la radioactivité spécifique du $P\beta$ de l'ATP est 50 fois plus petite que celle du $P\gamma$ (moyenne de 3 expériences). Les fragments mitochondriaux utilisés renferment donc très peu de myokinase.

pH optima pour l'échange ATP–P_i, pour l'hydrolyse de l'ATP et pour l'oxydation phosphorylante du β-hydroxybutyrate

Tracées en fonction du pH, les courbes de vitesse de l'échange ATP–P_i, de l'oxydation du β-hydroxybutyrate et de la phosphorylation couplée d'ADP ont en commun deux pics distincts, l'un à pH 6.5, l'autre à pH 7.3 (Fig. 1). L'échange ATP–P_i présente un pic principal à pH 7.3 et un pic secondaire à pH 6.5. L'oxydation du β-hydroxybutyrate, la phosphorylation d'ADP qui y est associée ainsi que le quotient de phosphorylation P/O présentent au contraire un pic principal à pH 6.5 et un pic secondaire à pH 7.3. Ces pics ne semblent pas résulter d'artefacts dûs à des concentrations limitantes en ATP, P_i et ADP; ces réactifs sont en effet présents dans le milieu à des concentrations 5 à 25 fois supérieures aux valeurs des constantes de

Michaélis correspondantes (déterminations personnelles et réf. 11, 22). HÜLSMAN ET SLATER[33] avaient déjà observé des valeurs maxima de quotients de phosphorylation à pH 6.3, 7.4 et 8.5 lorsque du glutamate était oxydé par des sarcosomes de coeur de boeuf.

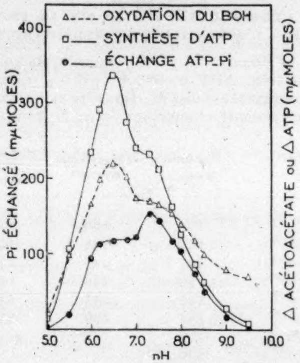

Fig. 1. Influence du pH sur la vitesse d'oxydation du β-hydroxybutyrate (BOH), sur le rendement de la phosphorylation associée et sur la vitesse d'échange ATP–^{32}P. P_i, 0.05 M; fragments sub-mitochondriaux, 120 μg N pour la mesure de l'échange ATP–^{32}P; fragments sub-mitochondriaux: 100 μg N pour la phosphorylation oxydative. Voir aussi MATÉRIEL ET MÉTHODES. Incubation, 15 min à 25°.

Trois pH optima caractérisent la vitesse d'hydrolyse de l'ATP: 6.5, 7.3* et 8.5. Ces valeurs de pH sont proches de celles trouvées par PURVIS[34], mais dans nos expériences l'addition de Mg^{++} n'est pas indispensable à la mise en évidence des pics d'activité de l'ATPase. Cette différence est sans doute liée au Mg^{++} intraparticulaire qui reste attaché aux fragments sub-mitochondriaux en quantités plus ou moins importantes suivant la méthode de préparation. SWANSON[35], MYERS ET SLATER[36, 37] ont également montré l'influence du pH sur l'ATPase des mitochondries entières de foie de rat.

Les réactions qui s'effectuent préférentiellement à pH 6.5 et à pH 7.3 sont communes à l'échange ATP–P_i, à l'ATPase et à la respiration. Au contraire, la (ou les) réaction qui se fait à pH 8.5 semble contrôler uniquement l'hydrolyse de l'ATP.

Effet de l'antimycine A et du cyanure sur l'état d'oxydo-réduction des transporteurs d'électrons dans des fragments sub-mitochondriaux

Si on ajoute à des fragments sub-mitochondriaux du cyanure en quantité suffisante pour stopper toute respiration (concn. finale $2 \cdot 10^{-3}$ M), les transporteurs d'électrons intraparticulaires: cytochromes a, a_3, b et c et flavoprotéine deviennent notablement réduits (Fig. 2). Il existe donc dans les fragments de mitochondries suffisamment de substrats endogènes pour assurer, en présence d'un inhibiteur respiratoire, une réduction importante des transporteurs localisés entre le DPN et le point d'action de l'inhibiteur. Cette réduction est accrûe si l'on emploie simultanément du β-hydroxybutyrate et du cyanure au lieu de cyanure seul (Fig. 2).

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* Le pic d'activité de l'ATPase à pH 7.3 est d'autant plus marqué que la concentration en EDTA est plus faible.

La présence d'antimycine A à une concentration inhibant complètement la respiration: 2 μg/ml (Tableau III), entraîne une réduction du cytochrome *b* et de la flavoprotéine qui est décelable mais qui peut être augmentée par l'addition de β-hydroxybutyrate (Fig. 3); cette augmentation dans le cas du cytochrome *b* est d'environ 30 %. Un tiers environ du cytochrome *b* est réduit (Fig. 3). lorsque l'antimycine A est utilisée à une concentration qui ralentit de moitié la respiration: 0.1 μg/ml (Tableau III).

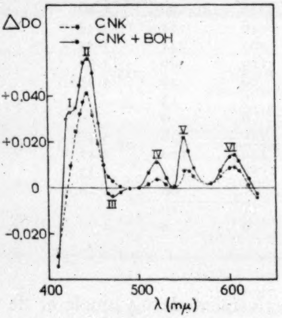

Fig. 2. Spectre différentiel entre l'état oxydé et l'état réduit de la portion de chaîne DPN ... cytochrome a_3. Les pics et la dépression correspondent à l'état réduit: I et II représentent les bandes de Soret des cytochromes *c*, *a* et a_3, III la flavoprotéine, IV et V les bandes β et α du cytochrome *c*, VI la bande α des cytochromes *a* et a_3. Le milieu d'incubation contient: fragments sub-mitochondriaux, 750 μg N; ATP pH 7.3, 0.0075 *M*; P_i pH 7.3, 0.003 *M* et suivant le cas KCN 0.002 *M*; β-hydroxybutyrate (BOH), 0.002 *M*. Volume final 2.7 ml. Température, 23°. Les lectures sont faites 5 min après l'addition de fragments sub-mitochondriaux au milieu d'incubation; à partir de ce moment l'état de réduction des transporteurs d'électrons dans les fragments est stable.

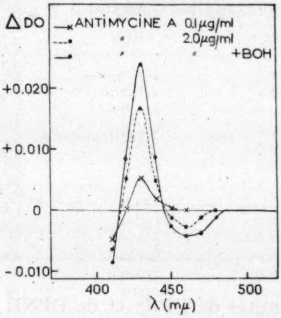

Fig. 3. Spectre différentiel entre l'état oxydé et l'état réduit de la portion de chaîne DPN ... cytochrome *b*. Le pic correspond à la bande de Soret du cytochrome *b* réduit et la dépression à la flavoprotéine. Le milieu d'incubation contient: fragments submitochondriaux, 750 μg N; ATP pH 7.3, 0.0075 *M*; P_i pH, 7.3, 0.003 *M* et suivant le cas de l'Antimycine A à la concentration indiquée et du β-hydroxybutyrate (BOH) 0.002 *M*. Volume final, 2.7 ml. Température, 23°. Par ailleurs les conditions sont identiques à celles données dans la légende de la Fig. 2.

En somme, lorsqu'un inhibiteur est ajouté à des fragments de mitochondries à une concentration capable de bloquer la respiration, la réduction subie par les transporteurs d'électrons intraparticulaires est soit totale soit subtotale, suivant la présence ou l'absence d'un substrat exogène oxydable dans le milieu d'incubation; la réduction n'est que partielle lorsque l'inhibiteur est utilisé à une concentration qui ralentit la respiration sans la bloquer.

L'existence de substrats endogènes oxydables dans les fragments sub-mitochondriaux pose, évidemment, la question de la proportion des formes oxydée et réduite des pyridine-nucléotides qui s'y trouvent. La détermination a été effectuée sur des fragments sub-mitochondriaux préparés à partir de 10 foies de rat et mis en incubation pendant 5 min à 25° avec de l'ATP 0.0075 *M* pH 7.3, de l'orthophosphate 0.0075 *M* pH 7.3 et de l'EDTA 0.0025 *M*, c'est-à-dire dans un milieu analogue à celui mis en oeuvre pour la mesure de l'échange ATP–P_i. Le DPN^+ et le DPNH étaient alors extraits et dosés comme il a été indiqué dans MATÉRIEL ET MÉTHODES.

TABLEAU III

EFFET DE CONCENTRATIONS CROISSANTES EN AMYTAL ET EN ANTIMYCINE A SUR L'OXYDATION DU β-HYDROXYBUTYRATE ET SUR LA PHOSPHORYLATION ASSOCIÉE

Conditions données dans MATÉRIEL ET MÉTHODES; pH, 6.5; Incubation 15 min à 25°.

Fragments submitochondriaux µg N	*Inhibiteur respiratoire*		*ATP synthétisé mµmoles*	*Acétoacétate formé mµmoles*	$\frac{P}{O}$
95	Amytal	0	280	200	1.40
		$0.03 \cdot 10^{-3}$ *M*	246	172	1.43
		$0.06 \cdot 10^{-3}$ *M*	207	144	1.44
		$0.12 \cdot 10^{-3}$ *M*	119	86	1.38
		$0.25 \cdot 10^{-3}$ *M*	59	46	1.28
		$0.50 \cdot 10^{-3}$ *M*	7	13	
		$1.00 \cdot 10^{-3}$ *M*	0	0	
100	Antimycine A	0	335	228	1.47
		0.05 µg/ml	240	170	1.41
		0.10 µg/ml	185	135	1.37
		0.20 µg/ml	71	60	1.18
		1.00 µg/ml	14	10	
		2.00 µg/ml	0	0	

Les quantités de DPN$^+$ et de DPNH sont respectivement de 0.5 µmole et de 0.2 µmole/g d'azote.

Dans les conditions de l'échange ATP–P_i et en l'absence d'inhibiteur respiratoire, l'état d'oxydo-réduction des transporteurs d'électrons de la chaîne DPN ... O_2 correspond approximativement à "l'état 1" tel qu'il a été défini par CHANCE ET WILLIAMS[6,38]. Ces transporteurs sont sous un état d'autant plus oxydé que leur potentiel d'oxydo-réduction est plus élevé. En particulier, il est probable que les cytochromes *a*, a_3 et *c* sont complètement oxydés tandis qu'une faible proportion de flavoprotéine et de cytochrome *b* se trouve sous un état réduit en raison de la proximité qui existe entre ces deux transporteurs et le DPNH.

Effet de l'amytal, de l'antimycine A, du cyanure et de l'anaérobiose totale sur l'échange ATP-P_i

La vitesse d'échange ATP–P_i a tout d'abord été mesurée lorsque les portions de chaîne: DPN ... "point d'action de l'amytal", DPN ... cytochrome *b*, DPN ... cytochrome a_3 étaient maintenus dans un état de réduction sub-totale ou totale par addition respective d'amytal, d'antimycine A, de cyanure ou de cyanure en présence de β-hydroxybutyrate ou encore par anaérobiose totale. Dans cette première série d'expériences, les inhibiteurs étaient ajoutés au milieu à des concentrations bloquant tout transfert d'électrons. Quel que soit l'inhibiteur respiratoire utilisé, la vitesse de l'échange ATP–P_i est diminuée (Tableau IV). Le ralentissement de l'échange est plus important à pH 7.3 qu'à pH 6.5; à pH 7.3 ,il est plus marqué lorsque la concentration en orthophosphate est plus faible. La présence de β-hydroxybutyrate n'accroît pas l'inhibition de l'échange due à l'addition de cyanure. Fait intéressant, l'amytal a une action inhibitrice au moins aussi grande, sinon plus, que l'antimycine A, le cyanure ou l'anaérobiose totale, ce qui est en accord avec les résultats que Löw *et al.*[15] ont obtenus avec des mitochondries entières.

Le comportement spécial de l'amytal a motivé une étude plus détaillée des rela-

TABLEAU IV

EFFET DE DIFFÉRENTS INHIBITEURS RESPIRATOIRES SUR L'ÉCHANGE ATP-^{32}P À pH 6.5 ET À pH 7.3

Conditions données dans MATÉRIEL ET MÉTHODES; Incubation 15 min à 25°. La concentration des inhibiteurs est celle requise pour arrêter toute respiration: KCN: 0.002 *M*; antimycine A, 2 µg/ml; amytal, 0.001 *M*. Du β-hydroxybutyrate (concn. finale 0.002 *M*) a été ajouté au milieu dans les expériences 2 et 3.

Exp.	*Fragments submitochondriaux µg N*	*Inhibiteur respiratoire*	P_i × 10^{-2} *M**	*Radioactivité spécifique de Pγ de l'ATP / Radioactivité spécifique de* P_i	
				A pH 6.5	*A pH 7.3*
1	110	Aucun	5.00	0.052	0.063
	110	Anaérobiose	5.00	0.041	0.047
2	100	Aucun	0.01	0.014	0.022
	100	KCN	0.01	0.012	0.007
3	100	Aucun	5.00	0.039	0.053
	100	KCN	5.00	0.035	0.043
4	66	Aucun	1.00	0.014	
	66	KCN	1.00	0.011	
5	120	Aucun	0.01	0.015	0.017
	120	Antimycine A	0.01	0.013	0.010
6	120	Aucun	5.00	0.038	0.050
	120	Antimycine A	5.00	0.031	0.036
7	130	Aucun	0.01	0.021	0.029
	130	Amytal	0.01	0.011	0.007
8	100	Aucun	5.00	0.054	0.066
	100	Amytal	5.00	0.020	0.033

* Les concentrations données sont celles au temps zéro d'incubation.

tions entre l'échange ATP–P_i et l'état d'oxydo-réduction des premiers transporteurs d'électrons de la chaîne respiratoire. On a réalisé des états de plus en plus réduits de la portion de chaîne DPN . . . cytochrome *b* en utilisant l'antimycine A à des concentrations croissantes allant de zéro jusqu'à la concentration nécessaire pour arrêter tout transfert d'électrons et l'on a mesuré dans chaque cas la vitesse de l'échange ATP–P_i. Lorsque la concentration en antimycine A est égale ou inférieure à 0.1 µg/ml, la vitesse de l'échange est augmentée significativement aussi bien à pH 7.3 qu'à pH 6.5 (Tableau V); l'augmentation est maximum (environ 20 %) pour une concentration en antimycine A, 0.1 µg/ml, qui est capable d'entraîner la réduction d'approximativement un tiers du cytochrome *b* présent dans les fragments sub-mitochondriaux (Fig. 3). Au delà de 0.2 µg/ml l'antimycine A exerce une action inhibitrice. Cette action inhibitrice ne semble pas être le résultat d'une dissociation de la phosphorylation oxydative (Tableau III); elle est vraisemblablement liée aux modifications de l'état d'oxydo-réduction de la portion de chaîne DPN . . .cytochrome *b*. En somme, la vitesse de l'échange ATP–P_i catalysé par des fragments sub-mitochondriaux à pH 6.5 et à pH 7.3 est maximum lorsque l'état d'oxydo-réduction des transporteurs d'électrons localisés entre DPN et cytochrome *b* est intermédiaire entre la réduction sub-totale et la quasi-oxidation. Aucun effet de stimulation n'a été mis en évidence dans le cas de l'amytal (Tableau V).

Effet de l'amytal, de l'antimycine A et du cyanure sur l'ATPase

L'activité de l'ATPase dans les fragments sub-mitochondriaux a été déterminée dans des conditions de réduction totale ou sub-totale des portions de chaîne: DPN . . .

cytochrome a_3, DPN . . . cytochrome *b*, DPN . . . "point d'action de l'amytal" suivant des méthodes analogues à celles utilisées pour l'étude de l'échange ATP–P_i.

WADKINS ET LEHNINGER[14] ont montré que le cyanure inhibe à pH 7.5 l'ATPase des fragments de mitochondries. En fait, les modifications d'activité d'ATPase qui résultent d'un état de réduction totale de la portion de chaîne DPN . . . cytochrome a_3 sont plus complexes et dépendent étroitement du pH. Dans nos conditions expérimentales, en présence de cyanure, l'hydrolyse de l'ATP est ralentie à pH 7.3 et accélérée au contraire à pH 6.5 et à pH 8.5 (Fig. 4). On observe des variations analogues si on remplace le cyanure par l'antimycine A: 2 µg/ml (Fig. 5). Par contre, l'addition d'amytal ralentit l'hydrolyse de l'ATP aux trois pH :6.5, 7.3 et 8.5 (Fig. 6).

L'activité de l'ATPase a également été mesurée pour des états de réduction partielle de la portion de chaîne: DPN . . . cytochrome *b*, ces états étant obtenus par l'addition d'antimycine A à des concentrations croissantes. A pH 7.3, l'activité

TABLEAU V

EFFET DE CONCENTRATIONS CROISSANTES EN AMYTAL ET EN ANTIMYCINE A SUR L'ÉCHANGE ATP–^{32}P À pH 6.5 ET À pH 7.3

Conditions données dans MATÉRIEL ET MÉTHODES; Incubation, 15 min à 25°.

Fragments mitochondriaux µg N	*Inhibiteur respiratoire*		P_i $\times 10^{-2}$ *M*	*P_i échangé*	
				A pH 6.5 mµmoles	*A pH 7.3 mµmoles*
80	Amytal	0	5.00	71	105
		$0.06 \cdot 10^{-3}$ *M*	5.00	60	102
		$0.12 \cdot 10^{-3}$ *M*	5.00	48	80
		$1.00 \cdot 10^{-3}$ *M*	5.00	35	66
100	Antimycine A	0	1.00	101	123
		0.05 µg/ml	1.00	105	135
		0.10 µg/ml	1.00	111	149
		0.20 µg/ml	1.00	86	108
		1.00 µg/ml	1.00	77	96
		2.00 µg/ml	1.00	74	87

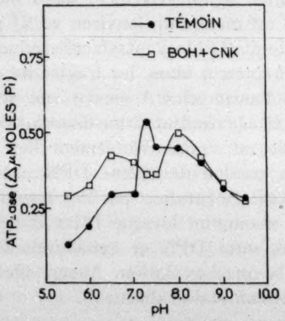

Fig. 4. Effet du cyanure sur l'ATPase à différents pH. KCN, $2 \cdot 10^{-3}$ *M*; β-hydroxybutyrate (BOH), $2 \cdot 10^{-3}$ *M*; fragments sub-mitochondriaux, 120 µg N. Voir aussi MATÉRIEL ET MÉTHODES. Incubation, 15 min à 25°.

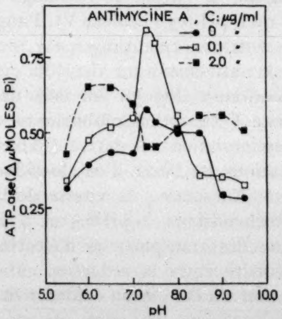

Fig. 5. Effet de l'antimycine A sur l'ATPase à différents pH. Conditions données dans MATÉRIEL ET MÉTHODES. Fragments sub-mitochondriaux, 120 µg N. Incubation, 15 min à 25°.

de l'ATPase est stimulée lorsque la concentration en antimycine A est égale ou inférieure à 0.1 μg/ml; elle est diminuée pour des concentrations en antimycine A supérieures à 0.2 μg/ml (Tableau IV, Fig. 5). Une réduction de plus en plus complète de la portion de chaîne DPN . . . cytochrome *b* entraîne donc des variations similaires de la vitesse d'échange ATP–P_i et de la vitesse d'hydrolyse de l'ATP à pH 7.3, ce qui est nettement mis en évidence dans la Fig .7. A pH 6.5 au contraire l'activité de l'ATPase augmente régulièrement en fonction de la concentration en antimycine A et se maintient à une valeur maximum pour des concentrations supérieures à 0.1 μg/ml (Tableau VI).

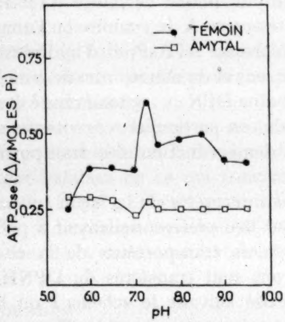

Fig. 6. Effet de l'amytal sur l'ATPase à différents pH. Conditions données dans MATÉRIEL ET MÉTHODES. Fragments sub-mitochondriaux, 130 μg N; amytal, 1 · 10^{-3} *M*. Incubation, 15 min à 25°.

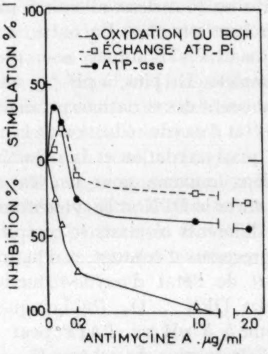

Fig. 7. Effet de concentrations croissantes en antimycine A sur les vitesses d'oxydation du β-hydroxybutyrate, de l'échange ATP–^{32}P et de l'hydrolyse de l'ATP à pH 7.3. P_i, 0.05 *M* pour la mesure de l'échange ATP–^{32}P, fragments sub-mitochondriaux: 100 à 120 μg N. Voir aussi MATÉRIEL ET MÉTHODES. Incubation, 15 min à 25°.

TABLEAU VI

EFFET DE CONCENTRATIONS CROISSANTES EN ANTIMYCINE A SUR LA VITESSE D'HYDROLYSE DE L'ATP A pH 6.5 ET A pH 7.3

Conditions données dans MATÉRIEL ET MÉTHODES; Quantité de fragments sub-mitochondriaux 100 μg N. Incubation, 15 min à 25°.

Antimycine A *μg/ml*	P_i *libéré à pH 6.5* *μmoles*	P_i *libéré à pH 7.3* *μmoles*
0	0.38	0.42
0.05	0.54	0.56
0.10	0.62	0.52
0.20	0.68	0.31
1.00	0.74	0.25
2.00	0.78	0.23

DISCUSSION

Les processus d'échange ATP–P_i et d'hydrolyse de l'ATP catalysés par des fragments sub-mitochondriaux de foie de rat sont caractérisés par 2 pH optima communs:

6.5 et 7.3. Deux pics d'activité à pH 6.5 et à pH 7.3 ont également été observés pour l'oxydation du β-hydroxybutyrate et pour la synthèse d'ATP qui y est associée. Ces résultats sont en accord avec la notion que l'échange ATP–P_i et l'hydrolyse de l'ATP sont des processus partiels de la phosphorylation oxydative[11]. Ils indiquent de plus que les différentes étapes phosphorylantes correspondant à l'oxydation du DPNH sont contrôlées, au moins, par deux réactions dépendantes du pH: l'une de ces réactions a pour pH optimum 6.5, l'autre 7.3.

Dans des conditions de réduction totale ou sub-totale des portions de chaîne DPN ... cytochrome a_3 ou DPN ... cytochrome *b*, l'échange ATP–P_i et l'hydrolyse de l'ATP sont tous deux ralentis à pH 7.3, tandis qu'à pH 6.5 l'échange est ralenti et l'hydrolyse stimulée. En outre, à pH 7.3, l'amytal inhibe l'échange ATP–P_i et l'hydrolyse de l'ATP autant, sinon plus, que l'antimycine A, le cyanure ou l'anaérobiose complète. De plus, à pH 7.3, les vitesses d'échange ATP–P_i et d'hydrolyse de l'ATP subissent des variations parallèles, de même sens et du même ordre de grandeur lorsque l'état d'oxydo-réduction de la portion de chaîne DPN ... cytochrome *b* évolue entre la quasi-oxydation et la réduction sub-totale; en particulier, ces vitesses sont toutes deux maxima pour un état moyen de demi-réduction des transporteurs localisés entre le DPN et le cytochrome *b*.

Ces différents résultats se complètent et sont interprétés de la façon suivante: (a) Les processus d'échange et d'hydrolyse qui ont lieu préférentiellement à pH 7.3 dépendent de l'état d'oxydo-réduction des premiers transporteurs de la chaîne respiratoire DPN ... O_2. (b) Lorsque des électrons sont transférés du DPNH au cytochrome *b* à pH 7.3, l'ATP peut être synthétisé suivant le schéma I ou bien suivant l'alternative du schéma II qui fait intervenir un transporteur T sous une forme de type "semi-quinonique" (voir INTRODUCTION).

Le cas général du schéma I est applicable au moins à la première étape phosphorylante DPN ... flavoprotéine. Il implique un transfert réversible de paires d'électrons, 2e, entre deux transporteurs T_1 et T_2 ainsi que la phosphorylation de l'un de ces transporteurs en un dérivé $T \sim P_i$. Cependant, les données expérimentales ne permettent pas de prévoir si T dans $T \sim P_i$ (réaction (1)) est oxydé ou réduit.

Dans le mécanisme de type "semi-quinonique" qui fait intervenir $T_{\text{semiquin}} \sim I$ (schéma II) ou $T_{\text{semiquin}} \sim P$ (schéma I), les électrons sont transférés par unités du DPNH vers un autre transporteur. Dans de telles conditions, la synthèse d'une liaison "riche en énergie" ($\Delta F \leq -8.000$ cal) nécessite que les potentiels d'oxydo-réduction du donneur et de l'accepteur d'électrons diffèrent d'au moins 0.35 V. Pour illustrer cette hypothèse on peut prendre comme exemple un transfert d'électrons-unités du DPNH au cytochrome *c*, chaque transfert étant associé à la synthèse d'une molécule d'ATP. Le transporteur T intervenant dans $T_{\text{semiquin}} \sim I$ ou $T_{\text{semiquin}} \sim P$ serait localisé entre le DPN et le cytochrome *c*; éventuellement il évoluerait de façon réversible de la forme "semi-quinonique" à la forme totalement réduite. Ainsi, en présence de I, le transfert d'électrons du DPNH à T_{semiquin} aboutirait à la formation de $T_{\text{red}} - I$ suivant la réaction:

$$T_{\text{semiquin}^{\cdot}} + I \xrightarrow{+\ e} T_{\text{red}} - I$$

le transfert ultérieur d'électrons de $T_{\text{red}} - I$ au cytochrome *c* potentialiserait la liaison entre T et I sous une forme "riche en énergie":

$$T_{\text{red}} - I \xrightarrow{-\ e} T_{\text{semiquin}^{\cdot}} \sim I$$

Des réactions analogues peuvent être envisagées en ce qui concerne la formation de $T_{semiquin} \sim P$.

En résumé, une molécule d'ATP se trouverait synthétisée chaque fois qu'un électron serait transféré du DPNH au cytochrome *c*. Le rendement de la phosphorylation d'ADP associée au transfert d'electrons du DPNH au cytochrome *c* est théoriquement le même dans le mécanisme "semi-quinonique" que dans celui qui fait intervenir des transferts de paires d'électrons: dans les deux cas, deux molécules d'ATP sont synthétisées quand deux molécules de cytochrome *c* sont réduites par une molécule de DPNH.

Nous n'avons pas retenu le schéma III dans cette discussion. Selon ce schéma, l'échange ATP–P_i et l'hydrolyse de l'ATP seraient indépendants de l'état d'oxydo-réduction de T ce qui n'est pas en accord avec nos résultats expérimentaux. Le schéma I ou l'alternative "semi-quinonique" des schémas I et II représentent donc des mécanismes possibles de la phosphorylation oxydative en relation avec des transferts d'électrons au niveau des premiers transporteurs de la chaîne DPN . . . O_2. Cependant, ces schémas ne sauraient être exclusifs; ils sont envisagés ici en tant qu'hypothèse de travail. D'autre part, d'autres schémas, en particulier le schéma III, restent valables en ce qui concerne au moins l'étape phosphorylante cytochrome *c* . . . O_2. Il n'y a effectivement aucun argument expérimental en faveur d'un mécanisme de la phosphorylation oxydative identique pour chacune des étapes phosphorylantes de la chaîne respiratoire DPN . . . O_2.

ADDENDUM

Des concentrations en antimycine A supérieures à 0.1 µg/ml qui ne modifient pas sensiblement le rapport P/O relatif à l'oxydation du β-hydroxybutyrate par l'oxygène diminuent significativement le rapport P/2 e relatif à l'oxydation du β-hydroxybutyrate par le cytochrome *c*. La diminution de l'effet stimulant sur l'échange ATP–P_i obtenue avec des concentrations en antimycine A supérieures à 0.1 µg/ml pourrait, de ce fait, être liée à une dissociation de la phosphorylation oxydative. Si cette hypothèse était vérifiée, ceci impliquerait que la phosphorylation (ou l'une au moins des phosphorylations) qui se produit entre le DPN et le point d'action de l'antimycine A peut faire intervenir un dérivé riche en énergie sous un état réduit $T_{red} \sim I$ (schéma II). Des expériences ultérieures doivent permettre de le préciser.

(Reçu le 23 août 1960)

REMERCIEMENTS

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RÉSUMÉ

1. On a étudié l'effet du pH et de l'état d'oxydo-réduction des transporteurs d'électrons de la chaîne DPN . . . O_2 sur des réactions partielles de la phosphorylation oxydative catalysées par des particules sub-mitochondriales.

2. Deux pH optima 6.5 et 7.3 caractérisent la vitesse d'oxydation du β-hydroxybutyrate, le rendement de la phosphorylation qui y est associée et la vitesse d'échange ATP–P_i. Trois pH optima 6.5, 7.3 et 8.5 caractérisent l'activité de l'ATPase.

3. Des états de réduction partielle, sub-totale ou totale de la chaîne respiratoire DPN . . . O_2 ou de portions de cette chaîne ont été obtenus grâce à l'emploi des inhibiteurs: amytal, antimycine A, cyanure ou grâce à l'anaérobiose complète.

4. Une réduction sub-totale ou totale des transporteurs d'électrons situés entre le DPN et le cytochrome *b* ou entre le DPN et le cytochrome a_3 entraîne à pH 6.5 une augmentation de l'activité de l'ATPase et une diminution de la vitesse d'échange ATP–P_1 alors qu'à pH 7.3 l'activité de l'ATPase et la vitesse d'échange sont toutes deux diminuées.

5. Un état de demi-réduction des transporteurs situés entre le DPN et le cytochrome *b* entraîne à pH 7.3 une stimulation de l'activité de l'ATPase et de l'échange ATP–P_1.

6. Les résultats obtenus suggèrent deux mécanismes possibles de phosphorylation oxydative pour le transfert d'électrons du DPNH au cytochrome *b*. Ces mécanismes ont été discutés en fonction des résultats expérimentaux.

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DYNAMICS OF THE PHOTOSYNTHESIS OF CARBON COMPOUNDS

I. CARBOXYLATION REACTIONS

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SUMMARY

Kinetic studies have been made of the rates of appearance of ^{14}C in individual compounds formed by *Chlorella pyrenoidosa* during steady state photosynthesis with $^{14}CO_2$. These rates have been compared with rates of CO_2 and ^{14}C disappearance from the gas phase during the same experiments.

The following results were obtained:

1. After the first few seconds, the rate of appearance of ^{14}C in compounds stable to drying on planchets at room temperature is 95 to 100 % of the rate of uptake of carbon from the gas phase.

2. After the first few seconds, the rate of appearance of carbon in compounds isolable by usual methods of paper chromatography constitutes at least 73 to 88 % of the rate of uptake of carbon from the gas phase. Compounds formed from the carbon reduction cycle via the carboxylation of ribulose diphosphate account for a least 70 to 85 % of the uptake, while carboxylation of phosphoenolpyruvic acid appears to account for at least another 3 %.

3. The induction period in the appearance of ^{14}C in stable compounds may be due to a reservoir of intracellular CO_2 and HCO_3^- or to some other volatile or unstable compound. If so, this reservoir contains no more than 1.5 μmoles of carbon, corresponding to about 7 sec carbon fixation in the experiment in which it was measured.

4. No other carboxylation reactions, such as the carboxylation of γ-aminobutyric acid, could be observed. The rate of labeling of glutamic acid after 5 min of exposure of the algae to $^{14}CO_2$ reached a maximum rate of about 5 % of the total uptake rate, but this labeling appears to be due to conversion of labeled intermediates formed from the carbon reduction cycle or phosphoenolpyruvic acid carboxylation.

5. The *in vivo* carboxylation of ribulose diphosphate in the light appears to be followed by conversion of the product to one molecule of phosphoglyceric acid, containing the newly incorporated $^{14}CO_2$ and one molecule of some other (kinetically distinguishable) three carbon compound. This reaction would be different from the one reported for the isolated enzyme system and the *in vivo* reaction in the dark, which produces two molecules of 3-phosphoglyceric acid.

Abbreviations: PGA or 3-PGA, 3-phosphoglyceric acid; PEPA, phosphoenolpyruvic acid; RuDP, ribulose 1,5-diphosphate; ATP, adenosine triphosphate; TPNH, reduced triphosphopyridine nucleotide.

INTRODUCTION

Much of the biochemical pathway through which carbon dioxide is reduced during photosynthesis in algae has been established[1-3]. A principal feature of this pathway is the carbon reduction cycle. A simplified version of this cycle is given in Fig. 1, which shows the key steps.

To map these paths, CALVIN *et al.*[3-10] gave radioactive compounds, such as $^{14}CO_2$ and $KH_2{}^{32}PO_4$, to photosynthesizing plants. The plants made various reduced organic compounds from these labeled substrates. They were then killed and the soluble compounds were extracted from the plant material and analyzed by two-dimensional paper chromatography and radioautography. The compounds were identified and their radioactive content determined. From the amount and location of radioactive elements within compounds following exposures of the plants for various lengths of time and under various environmental conditions, biochemical pathways were followed.

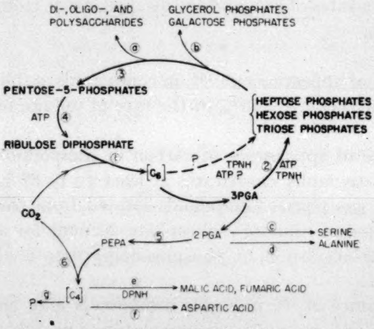


Fig. 1. Carbon reduction cycle (simplified version). (1) Ribulose diphosphate reacts with CO_2 to give an unstable six carbon compound which splits to give two three carbon compounds. At least one of these is 3-phosphoglyceric acid. The other three carbon compounds might be either 3-PGA, as it is known to be in the isolated enzyme system, or some other three carbon compound such as a triose phosphate (dashed arrow). (2) PGA is reduced to triose phosphate with ATP and TPNH derived from the light reaction and water. (3) Various condensations and rearrangements convert the triose phosphates to pentose phosphates. (4) Pentose phosphate is phosphorylated with ATP to give ribulose diphosphate. Further carbon reduction occurs *via* conversion of PGA to phosphoenolpyruvic acid, (5), and carboxylation, (6), to form a four carbon compound (probably oxaloacetic acid). Reactions leading to the formations of some of the secondary intermediates in carbon reduction are shown by the arrows lettered a through g.

In the present study we have extended our information about these pathways by more precise control of the environmental conditions during exposure of the plants to tracers. At the same time we have made measurements of the rate of entry of tracer into the plant and of the rate of appearance of the tracer in specific compounds.

We sought answers to the following questions: (a) How much of the total carbon taken up by the plants enters the metabolic network via carboxylation of ribulose diphosphate (reaction 1)? (b) How much of the total carbon taken up enters by carboxylation of PEPA (reaction 6)? (c) Are any other carboxylation reactions, such as the carboxylation of γ-aminobutyric acid[11], of any importance in steady state photosynthesis? (d) Does the carboxylation of ribulose diphosphate *in vivo* lead to one product only (PGA) or does it lead to two products (PGA and some other 3-carbon compound)?

"Steady state photosynthesis" as used in this paper, is defined as a condition under which unicellular algae are carrying out the reaction of photosynthesis, are synthesizing all of the normal cell constituents, and are growing and dividing at constant rates during the course of the experiment. Moreover, the rates of photosynthesis in experiments which will be reported here were between 30 and 80 % of the maximum rates at which these algae are capable of photosynthesizing at room temperature.

EXPERIMENTAL

Plant material

The plants used in all experiments were the unicellular green algae, *Chlorella pyrenoidosa*, raised in continuous automatic culture tubes as described previously[3]. The algae were raised and harvested as a 0.5 % (volume wet packed cells/volume) suspension. The algae were centrifuged from the culture medium and then suspended in a special nutrient solution (described later). This suspension (80 ml) was placed in the illumination chamber of the steady state apparatus.

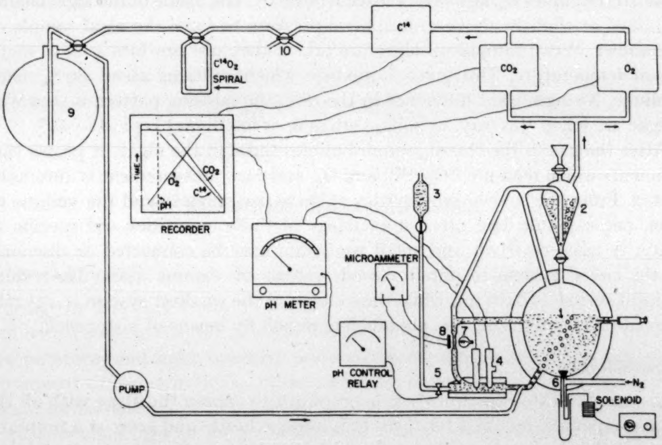


Fig. 2. Steady state apparatus. (1) algae chamber, (2) water or nutrient solution reservoir, (3) acid or base reservoir, (4) pH electrodes, (5) solenoid operated pH control valve, (6) solenoid operated sampling valve, (7) small lamp, (8) photovoltaic cell, (9) large gas reservoir, (10) four-way stopcock.

Steady state apparatus

In the steady state apparatus, shown schematically in Fig. 2, a stream of gas (1 to 2 % CO_2 in air) is cycled through a closed system. The gas is bubbled through the 0.5 % or 1.0 % suspension of algae (80 ml) at a rate of approximately 1 l/min. Gas and liquid mix rapidly in the algae chamber, which is 3/8″ thick and 4″ in diameter (inside dimensions). The algae chamber is illuminated from both sides by G.E. RSP2 photospot incandescent lights through an infrared absorbing glass in a water bath, or in some experiments from one side by an incandescent lamp and from the other side by a bank of eight 8″, 6 W fluorescent lamps (blue and cool white). In either case, the voltage to the incandescent lamps is adjusted just to give light saturation of the oxygen evolution rate. The algae chamber is water jacketed, and the water is circulated in a thermostated bath. The temperature of this bath is set so that during steady state photosynthesis the temperature indicated by the thermometer in the algae suspension reads 25°.

The algae chamber is connected to a side loop through which the algae suspension is made to circulate by the flow of gas into the chamber. A beam from a small lamp passes through a window in the side loop to a photovoltaic cell which measures the light absorption and hence the density of the algae. Electrodes in the side loop measure pH, which is recorded on a multipoint recorder. The pH meter output is also connected to a control relay which, through the activation of a solenoid-operated valve, can cause acid or base from a reservoir to be added in small volumes to the algal suspension. Another reservoir within the closed system contains distilled water or nutrient solution, which can be added to the algal suspension to dilute it to the selected concentration as the algae grow.

A solenoid-operated sampling valve at the bottom of the chamber permits one to take 1-ml samples rapidly (every 2 sec if desired). The inside of the algal chamber is maintained at slightly above atmospheric pressure to force the algal sample out of the chamber. When samples of algae are taken, they are run into 4 ml of methanol at room temperature. This gives a mixture which contains about 80% methanol by volume. No significant difference in the resulting labeling pattern is seen whether the algae are killed this way, in boiling ethanol, or in ethanol kept at −40°.

After the gas in the closed system bubbles through the algae, it passes through instruments which measure CO_2, ^{14}C, and O_2, and each measurement is automatically recorded. From the known sensitivities of these instruments and the volume of the system, one can calculate rates of exchange of these quantities and specific radioactivity. A large reservoir and small reservoirs may be connected or disconnected from the closed system to obtain closed systems of various sizes. The volume of the largest system is 6400 ml, while the volume of the smallest system is 435 ml. The system can be open during the pre-labeling period by means of a stopcock.

Nutrient solution

For steady state experiments it is necessary to supply the algae with all the inorganic compounds required for them to photosynthesize and grow at a normal rate. Unfortunately, the nutrient solution in which they are usually grown in the laboratory contains quantities of salts which make impossible an adequate separation of labeled compounds by two-dimensional paper chromatography. Therefore, the algae are suspended in much more dilute nutrient solutions of which that in Table I is typical.

TABLE I

STARTING NUTRIENT SOLUTION FOR STEADY STATE EXPTS. 18 AND 28

$(NH_4)_2HPO_4$	40 mg/l
$MgSO_4 \cdot 7H_2O$	20 mg/l
NH_4Cl	20 mg/l
KNO_3	20 mg/l
ARNON'S A-4 solution of trace elements plus $CoCl_2 \cdot 6H_2O$ (40 mg/l) and MoO_3 (15 mg/l)[12]	1 ml/l
Fe^{++}-versenol solution to give 90 m*M* Fe^{++}	1 ml/l
NH_4VO_3 (23 mg/l)	1 ml/l

This medium was adequate to maintain nearly a constant rate of photosynthesis in experiment steady state No. 18. In other experiments, such as steady state 28, the algae growing under steady state conditions would in time exhaust the supply of ammonium ion contained in this medium. However, it has been observed that as the algae take up ammonium ion, the pH of the medium tends to decrease, presumably due to the exchange into the medium of hydrogen ions for ammonium ions. Therefore, dilute NH_4OH was added to the algae suspension automatically by the pH control system, thereby maintaining constant pH. At the same time ammonium ion concentration was maintained approximately constant. The nutrient solution for pH control was diluted by trial and error until its addition kept the algae density constant. To it were added other inorganic ions in a ratio to the ammonium ion which was estimated to provide the algae with an adequate level of these ions for growth for a limited period. The resulting pH control medium used in steady state experiment 28 is shown in Table II.

TABLE II

CONTROL MEDIUM USED IN STEADY STATE EXPERIMENT 28

$(NH_4)HPO_4$	6.6 mg/l
$(NH_4)_2SO_4$	6.6 mg/l
NH_4OH	0.55 mg/l
$FeCl_3 \cdot 6H_2O$	5.0 mg/l
KCl	8.0 mg/l

Trace elements as in starting medium

Administration of ^{14}C

During the first part of the experiment the algae are kept photosynthesizing in the light with a constant supply of 1.5 to 2 % unlabeled CO_2 in air for 0.5–1 h. Constant pH, temperature, and light intensity are maintained during this time, and during the subsequent exposure to $^{14}CO_2$. In the experiments reported here the pH was kept at 6. Rate measurements of CO_2 uptake and O_2 evolution are made by making the closed system small, 435 ml for a few minutes, and observing the rate of change of CO_2 and O_2 tensions as indicated on the recorder. The closed gas system is made large again, and at zero time, $^{14}CO_2$ is added to the system by turning a stopcock. At the same instant a solution of $NaH^{14}CO_3$ is injected directly into the algal suspension. The amount and specific radioactivity of the injected bicarbonate solution is so calculated that it will immediately bring the specific radioactivity of the dissolved CO_2 and bicarbonate already present in the algal suspension to its final value. This is the specific radioactivity which will obtain for all the CO_2 and bicarbonate in the gas and liquid phases of the closed system after complete equilibration has occurred. An example of this calculation is given in Table III. Samples of the algae suspension of uniform size are taken every 5 or 10 sec for the first few minutes, and then less frequently for periods up to 1 h. Each sample is taken directly into 4.0 ml of methanol (room temperature) in a centrifuge tube (preweighed). Sample tubes are reweighed to give the sample size (± 1 %). After an hour at room temperature, the samples are centrifuged and the 80 % methanol extract removed. 1 ml of methanol is added to the residue and stirred a few minutes, then 4 ml water is added and the mixture

TABLE III

CALCULATION OF $^{12}C + ^{14}C$ FOR STEADY STATE EXPT. 18

	Volume	%CO_2	µmoles	µ$C^{14}C$	Specific activity
A Gas phase at start	895	1.6	585	0	0
B $^{14}CO_2$ loop	72		156	3767	
C Dissolved CO_2, HCO_3	125*		81.6		0
D $NaH^{14}CO_3$ injected			40.8	607.5	
Total	1092		863	4375	5.07 µC/µmole
C + D			122.4	607.5	4.95 µC/µmole

* Effective volume.

warmed at 60° for 10 min. After centrifugation and a further extraction with 1 ml of water, the combined clear extracts are concentrated at reduced pressure at below room temperature. The concentrated extract, or an aliquot portion thereof, is transferred quantitatively to the paper chromatogram and analyzed in two dimensions (phenol–water, butanol–propionic acid–water) as in earlier work[5]. The location of the radioactive compounds on the chromatograph is found by radioautography with X-ray film. When necessary, overlapping phosphate esters are eluted, treated with phosphatase and rechromatographed.

Determination of radioactivity in compounds

The amounts of radiocarbon in each compound of interest on the chromatograms from each sample is measured with a Geiger-Mueller tube. The paper chromatogram is placed on top of the radioautograph, which rests on a horizontal light table, so that the darkened areas of the film may be seen through the paper. The Geiger-Mueller tube has a Mylar window, gold-sputtered for conductivity, but transparent and thin (less than 1 mg/cm^2) to permit the passage of ^{14}C beta particles. This tube has an effective counting area of uniform sensitivity of about 17 cm^2. The top of the tube is transparent plastic so that paper and radioautograph may be viewed through the top of the tube. Thus the counting area of the tube may readily be placed in position over the radioactive compound on the paper. If the radioactive area is more than 4 cm across, or if it contains more than 20,000 counts/min (as counted by this tube on the paper), the radioactive area is divided into smaller areas which are counted one at a time (with the remainder of the spot covered by cards). The counting gas used is helium–isobutane (99:1). The counting voltage is about 1300 V. The sensitivity of the counter for ^{14}C beta particles in an infinitely thin layer on an aluminum planchet is about one count/3.1 disintegrations. However, only about one-third of the beta particles escape from the paper (Whatman No. 4) and the actual sensitivity of this tube for ^{14}C in compounds on the paper is about 1/11.2. These sensitivities were determined by comparison of counts from three aliquot portions of a known ^{14}C labeled solution: (a) chromatographed on paper, (b) dried on a planchet, and (c) placed in a scintillation counter with an internal standard. The radioactivity of each compound is counted on each side of the paper and an average is taken of the counts from the two sides. Comparison with determinations of radioactivity of compounds quantitatively eluted and placed on planchets indicates that this method of counting gives an accuracy of ± 5 %.

Rate measurements

Gas exchange: Measurements of the rates of CO_2 uptake, ^{14}C uptake and O_2 evolution by the photosynthesizing algae are made by taking the slopes of the three traces on the recorder. In order to obtain accurate readings in 10 min or less, the total effective gas volume of the closed circulating system is made small, about 435 ml. With 80 ml of 0.5 % algal suspension in the system the resulting change in O_2 or CO_2 pressure is about 0.5 % in 10 min in a typical experiment. This corresponds to a rate of 22 μmoles of gas exchange/min/ml of wet packed algae. The response of the Beckman Infrared Analyzer, model 15 A, used in these experiments is not completely linear in the range used (0 to 2.0 % CO_2) so that a correction based on a previously obtained calibration curve is applied to the CO_2 uptake curve plotted on the recorder. The response of the A. O. Beckman oxygen analyzer is essentially linear in the range used (19 to 21 %). The level of ^{14}C is plotted on the recorder as millivolts response of the Applied Physics Corpn.'s Vibrating Reed Electrometer to the ionization chamber (volume 118 ml, $R = 10^9$ ohms). From the known calibration of the ionization chamber this reading can be directly converted to μC of ^{14}C. From the $^{12}CO_2$ reading and the ^{14}C reading the specific radioactivity of the CO_2 may be calculated at all times during the experiment. This specific radioactivity is used to convert the rates of change of radioactivity in the system to rates of change of what we shall call "^{14}C" throughout this paper. For convenience of expression and calculation, this ^{14}C will be expressed in μmoles and represents the amount of ^{12}C and ^{14}C corresponding to a given measured amount of radioactivity in the CO_2 administered to the algae at any time during the experiment.

Total fixation in algae: In some experiments, small aliquot portions of each sample of algal material, taken and killed in alcohol during the course of the experiment, are spread in a thin layer on planchets with acetic acid, dried, and counted. The amount of ^{14}C found at each time of exposure of the algae to $^{14}CO_2$ is plotted and the slope of the curve drawn through these points gives the rate of appearance of ^{14}C in stable compounds in the plant.

Fixation of ^{14}C in compounds found on the paper chromatograph: After the ^{14}C in individual compounds found on the paper chromatogram has been measured, the amounts are sometimes totaled for each sample up to one minute, and a rate of appearance of ^{14}C in these compounds is calculated.

RESULTS

Steady state Expt. 18

The rates of exchange of gases before, during and at the end of the experiment are shown in Table IV. We shall take 15.5 μmoles/min as an average value for uptake of carbon during the experiment.

Aliquot portions of the samples were dried on planchets and their radioactivity was counted. When results of these counts were plotted *versus* time of sampling, the rate of fixation of ^{14}C into compounds stable to drying on the planchets was found to be about 15 μmoles/min (Fig. 3).

After chromatographic separation of the compounds, radioautographs, of which Fig. 4 is typical, were obtained. The radioactivity of each compound in each sample was determined and the total radiocarbon found in the various compounds

TABLE IV

RATES OF GAS EXCHANGE IN STEADY STATE EXPT. 18

All rates are given in μmoles/ml of wet packed algae

	Carbon dioxide	^{14}C*
Initial rate	17.9	
During experiment	16.6	15.1
Final rate	14.1	13.7

* See section *Methods of measurements of rate of gas exchange* for explanation of expression of ^{14}C in μmoles. In theory the value for ^{14}C and CO_2 should be the same. The difference is a reflection of inaccuracy in measurement of the slopes, especially CO_2.

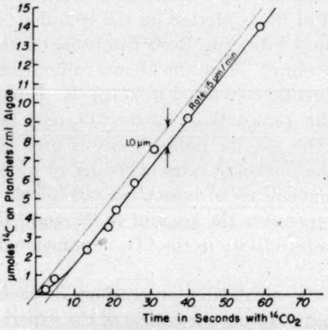


Fig. 3. Appearance of ^{14}C in stable compounds (dried on planchets) in *Chlorella pyrenoidosa vs.* time of photosynthesis with $^{14}CO_2$.

Fig. 4. Radioautograph of chromatogram of *Chlorella pyrenoidosa* after 2 min photosynthesis with $^{14}CO_2$.

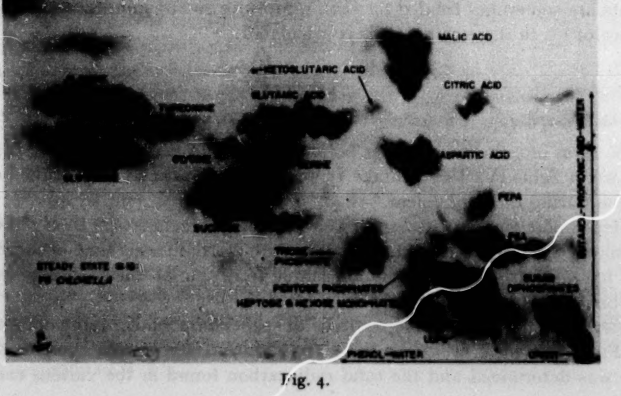

FUMARIC ACID
MALIC ACID
α-KETOGLUTARIC ACID
CITRIC ACID
GLUTAMIC ACID
THREONINE
GLYCINE
ASPARTIC ACID
SUCROSE
PEPA
PGA
TRIOSE PHOSPHATE
PENTOSE PHOSPHATES
HEPTOSE & HEXOSE MONOPHATES
SUGAR DIPHOSPHATES
STEADY STATE
PS CHLORELLA
PHENOL-WATER
ORIGIN
BUTANOL-PROPIONIC ACID-WATER

Fig. 4.

was plotted against time (Fig. 5). The maximum slope of the curve in Fig. 5 is 13 µmoles. This is a lower limit for the rate of appearance of ^{14}C in soluble compounds which are also stable to chromatography. It does not take into account other compounds, too weakly radioactive to be counted, or "lost" from the front of our chromatograms. (In order to obtain good separation of phosphate esters we customarily allow the phenol–water solvent to drip from the ends of the chromatograms. Small amounts of labeled fatty material are lost in this way.)

After 30 sec, appreciable amounts of radioactivity are passing through the extractable precursor compounds seen on the chromatograms into non-extractable substances, which are not seen on the chromatograms. Consequently the rate of appearance of ^{14}C in compounds on the paper decrease.

During the first ten seconds, the rate of appearance of ^{14}C in stable compounds is less than the maximum rate during the subsequent time. This could be ascribed to mixing time of the added $H^{14}CO_3^-$ with the $H^{12}CO_3^-$ present initially, or alternately to the presence of an intermediate pool of either HCO_3^- or some other unstable or volatile compound. Such a compound would precede the stable soluble compounds in the fixation pathway. The size of this "pool", if it exists, cannot be greater than the difference between the fixation curve after 10 sec and a line of the maximum slope drawn through the origin (see Figs. 3 and 5). This is no more than 1.0 to 1.5 µmoles, which is equal to the carbon fixed in 4 to 6 sec in this experiment. A calculation of the amount of HCO_3^- which would be found inside algae cells in a volume of 1 ml with an internal pH of 7 in equilibrium with 1.7 % CO_2 gives a value of about 1 to 1.5 µmoles, depending on the volume available inside the cells. It seems to us to be not unreasonable to suppose that this "pool" is merely intracellular CO_2 and HCO_3^- but it does not matter to the subsequent argument whether it is this or some other unstable or volatile substance.

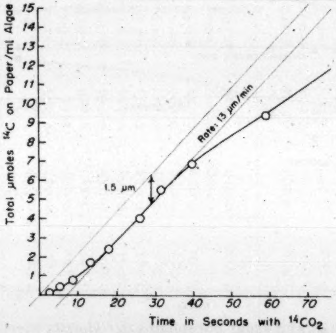


Fig. 5. Appearance of ^{14}C in compounds on chromatograms prepared from *Chlorella pyrenoidosa vs.* time of photosynthesis with $^{14}CO_2$.

From the measured rates of uptake of CO_2 and ^{14}C and from the rates of appearance of ^{14}C in stable compounds these experimental findings may be listed: (a) The appearance of ^{14}C in stable, nonvolatile compounds, after the first 10 sec of exposure of the plant to $^{14}CO_2$, is equal to the rate of total uptake of $^{14}CO_2$ within experimental error. (b) During the period between 10 and 30 sec exposure to $^{14}CO_2$, the appearance of ^{14}C in individual compounds which can be isolated by our methods of paper chromatography, is equal to at least 85 % of the rate of total uptake. (c) If there is a pool of CO_2, HCO_3^- or other unstable or volatile compound lying between administered CO_2 and stable compounds in the fixation pathway, its amount is not more than 1.0 to 1.5 μmoles (4 to 6 sec fixation) and it is essentially saturated after 10 sec.

Let us next consider the question of how much of this fixed ^{14}C must pass through the PGA pool.

In Fig. 6 are shown the labeling curves of some of the more rapidly labeled compounds and groups of compounds. By 3 min, compounds of the carbon reduction cycle are essentially saturated with radiocarbon. Secondary intermediates such as sucrose, malic acid, and several amino acids are not saturated until longer times (5 to 30 min). In order to evaluate the importance of the fixation pathway leading through PGA, we have tabulated the actual measurements of ^{14}C found in compounds during the first minute (Table V). The ^{14}C found in all those compounds derived from PGA without further carboxylation (see Fig. 1) is added together (T_1). Compounds labeled by C_3–C_1 carboxylation are totaled separately (T_2). Since three of the carbon atoms in these compounds are derived from PGA, their total radioactivity is multiplied by a factor which is 3/4 times the degree of saturation of the PGA, which is presumed to be the same as that of their immediate precursor, namely, PEPA. (The saturation curves for PGA and PEPA are in fact very similar in this and other experiments.) The sum of T_1 and T_2f, representing measured ^{14}C derived from the primary reaction which forms PGA, is plotted in Fig. 7. Again the "pool" of HCO_3^- or other volatile or unstable compound is about 1 μmole and in this case it must precede PGA in the chain of reactions. Where one draws the curve of maximum slope through these points is somewhat arbitrary, but the maximum rate of appearance of ^{14}C in these compounds falls somewhere between 11 and 13 μmoles/min. Thus on the basis of the appearance of ^{14}C in these extractable, stable compounds alone, at least 70 to 85 % of all carbon fixed during photosynthesis (measured externally) is incorporated via the carbon reduction cycle. It must be emphasized

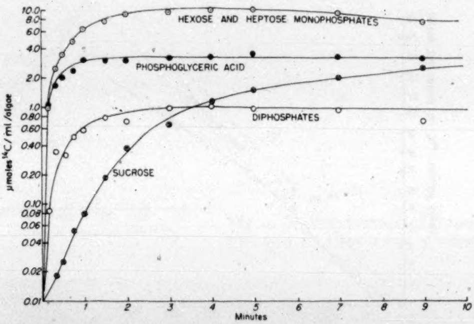


Fig. 6. Appearance of ^{14}C in PGA and sugar phosphates in *Chlorella pyrenoidosa vs.* time of photosynthesis with $^{14}CO_2$.

TABLE V

^{14}C (μMOLES/ML ALGAE) IN PHOTOSYNTHESIS INTERMEDIATES DURING FIRST MINUTE WITH $^{14}CO_2$

Steady State *Chlorella* 18.

Compound	*Time in seconds of $^{14}CO_2$*									*Steady state value*	
	2.5	*5*	*8.5*	*13*	*18*	*26*	*31.5*	*40*	*59*	*µmoles carbon*	*µmoles compound*
PGA	0.063	0.220	0.466	0.750	0.983	1.38	1.72	1.92	2.29	3.0	1.0
PEPA	0.0084	0.0083	0.026	0.041	0.047	0.093	0.114	0.132	0.170	0.2	0.07
Hexose monophosphate	0.017	0.063	0.205	0.429	0.677	1.14	1.60	1.99	2.73	5.5	0.9
Heptose monophosphate	0.011	0.027	0.080	0.157	0.247	0.406	0.617	0.766	0.955	1.8	0.26
Pentose monophosphate	0.0021	0.0096	0.011	0.024	0.035	0.054	0.074	0.067	0.133	~ 0.2	~ 0.04
Triose monophosphate	0.0069	0.0139	0.038	0.063	0.084	0.144	0.174	0.215	0.283	~ 0.8	~ 0.3
Diphosphates except ribulose	0.0041	0.013	0.026	0.044	0.053	0.093	0.138	0.160	0.203	~ 0.54	—
Ribulose diphosphate	0.0045	0.0129	0.028	0.047	0.057	0.100	0.150	0.190	0.225	0.36	0.07
Alanine	—	0.025	0.024	0.049	0.075	0.170	0.257	0.397	0.869	~ 15	~ 5
Serine	—	0.015	0.012	0.016	0.013	0.027	0.039	0.053	0.120	1.2	~ 0.4
Sucrose	—	—	—	—	0.0080	0.019	0.021	0.035	0.094	> 3.5	> 0.3
UDPG	—	—	—	0.0090	0.019	0.048	0.108	0.170	0.308	> 2.0	—
Total (T_1)	0.117	0.407	0.916	1.629	2.298	3.674	5.012	6.085	8.380		
Aspartic acid	—	—	0.0070	0.015	0.025	0.063	0.091	0.131	0.299	2.0	0.5
Fumaric acid	—	—	0.0020	0.0030	0.0080	0.013	0.025	0.050	0.091	0.7	0.18
Malic acid	—	0.0066	0.018	0.040	0.063	0.152	0.194	0.271	0.480	2.1	0.5
Total (T_2)		0.007	0.027	0.058	0.096	0.228	0.310	0.452	0.870		
x = PGA saturation	0.021	0.073	0.152	0.250	0.327	0.461	0.575	0.635	0.765		
f = 3x/4	0.016	0.055	0.116	0.158	0.246	0.347	0.432	0.476	0.574		
$T_2 \times f$	—	—	0.002	0.009	0.023	0.078	0.135	0.216	0.500		
Grand total = $T_1 + T_2f$	0.117	0.412	0.918	1.638	2.321	3.752	5.147	6.301	8.880		
$T_2 - T_2f$	—	—	0.025	0.049	0.073	0.150	0.175	0.236	0.370		

that this percentage is a lower limit based only on absolute measurements of identified compounds.

A lower limit for the amount of carbon incorporated via C_1 plus C_3 carboxylation is obtained by plotting T_2—T_2f (Fig. 7). The minimum rate of this incorporation is about 0.4 μmoles/min/ml algae, or about 3 % of the total. Note that this value is for the actual introduction of CO_2 and does not include the carbon derived from PGA (T_2f). The rate of incorporation of ^{14}C into these three compounds thus accounts for about 4 times 3, or 12 % of the total in this experiment. Other experiments indicate that the relative contribution of C_3–C_1 carboxylation varies considerably and tends to be higher (up to 3 times that reported in this case) when the rate of CO_2 fixation is greater and when amino acid synthesis is more rapid. In addition to the three compounds listed here, other substances may be derived in part from C_1–C_3 carboxylation, such as glutamic acid and citric acid, discussed below.

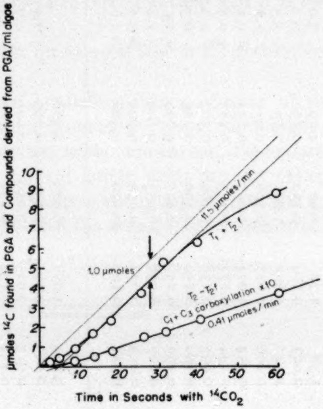


Fig. 7. Appearance of ^{14}C in compounds derived from PGA and in compounds derived from $C_1 + C_3$ carboxylation in *Chlorella pyrenoidosa vs.* time of photosynthesis with $^{14}CO_2$.

While at least 73 % of the total rate of fixation of carbon has thus been shown to be due to the carbon reduction cycle and C_1–C_3 addition, there is no indication of any other significant fixation pathway. In Fig. 8 the ^{14}C found in glutamic acid and in citric acid is shown. Could this labeling of glutamic acid be the result of a carboxylation of γ-aminobutyric acid? The maximum rate of labeling of glutamic acid and in citric acid is shown. The maximum rate of labeling of glutamic acid is about 0.7 μmoles/min or 4.5 % of all ^{14}C fixed. Since this rate is found between 5 and 20 min, it probably represents labeling of all five carbon atoms of glutamic acid, because the precursors are surely at least partially labeled after 5 min. The labeling due to carboxylation reaction would be expected to begin during the first 30 sec, if one is to judge by the other known carboxylation reactions which were discussed earlier. Yet, after the first 31.5 sec, the glutamic acid contains only 0.02 μmoles of ^{14}C. Between 40 and 60 sec, its labeling rate is only 0.2 μmoles/min. Moreover, γ-aminobutyric acid itself would have to be synthesized from CO_2 (by some as yet unknown route), if it were a precursor to glutamic acid, and would have to be appreciably labeled by the time glutamic acid reaches its maximum labeling rate. Yet we can detect no radiocarbon in γ-aminobutyric acid in this experiment or in others of this series, even after the algae have been exposed to $^{14}CO_2$ for 10 min. Clearly, little if any of the labeled glutamic acid formed in our experiments is made by carboxylation of γ-aminobutyric acid. Rather, it must arise from other intermediate substances such as those formed by the two carboxylation mechanisms already discussed.

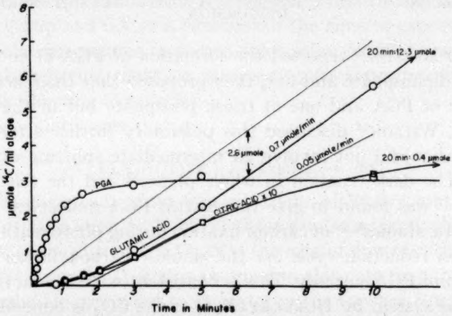


Fig. 8. Appearance of ^{14}C in PGA, glutamic acid and citric acid in *Chlorella pyrenoidosa vs.* time of photosynthesis with $^{14}CO_2$.

Note, however, that the rate of labeling of citric acid is by far too small to permit it to be the precursor of the labeled glutamic acid in any sequence such as:

$$\text{oxaloacetic acid} + \text{acetyl coenzyme A} \longrightarrow \text{citric acid} \rightarrow\rightarrow\rightarrow$$
$$\alpha\text{-oxoglutaric acid} + CO_2 \longrightarrow \text{glutamic acid}$$

Steady state Expt. 28

All the results described thus far were obtained in an experiment (steady state 18) in which the nutrient solution, though not automatically replenished, was sufficient to maintain the rate of photosynthesis at a nearly constant level during the course of the experiment. The results of steady state Expt. 28, in which the nutrient solution was replenished during the course of the experiment led to the same conclusions.

TABLE VI

COMPARISON OF STEADY STATE EXPERIMENTS 18 AND 28

Experiment	*CO_2 uptake µmoles/min/ml algae*	*Rate of appearance of ^{14}C in compounds on chromatograms (20–40 sec)*	*RuDP saturation at 40 sec*	*PGA residual* carbon saturation according to*	
				Reaction D	*Reaction L*
18	15.5	13	0.53	0.57	0.46
28	19.5	17–18	0.38	0.43	0.28

* See subsequent discussion for explanation of the term "residual". The degree of saturation at 40 sec is obtained by dividing the measured value of ^{14}C in the compound at 40 sec by the saturation level of ^{14}C in the compound (or residual atoms) after 10 min exposure of the algae to $^{14}CO_2$.

These results are summarized and compared with steady state Expt. 18 in Table VI. Though not shown in the table, the maximum rate of appearance of ^{14}C in observable compounds derived from the carboxylation reaction leading to PGA (the carbon reduction cycle) was 70 to 90 % of the externally measured rate of ^{14}C uptake.

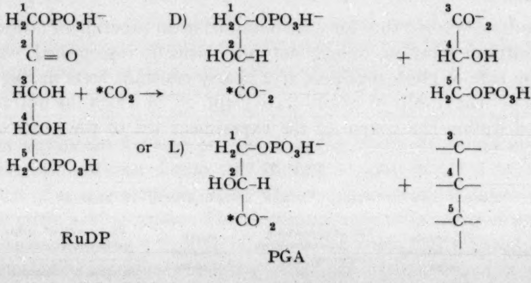
DISCUSSION

When Calvin and Massini[13] reported the formation of PGA in an overall reaction requiring ribulose diphosphate and CO_2 they proposed that the reaction in the light gave one molecule of PGA and one of triose phosphate but in the dark gave two molecules of PGA. Wilson[14] discussed this possibility further after it was realized that the carboxylation did not involve an intermediate splitting of the ribulose to triose and diose. The dark reaction in whole plants[15] and the reaction in isolated enzyme systems[16, 17] was found to give rise to two PGA molecules. Also, it is clear from previous kinetic studies[1, 18] of carbon fixation during photosynthesis that the ^{14}C entering the carbon reduction cycle *via* the ribulose carboxylation passes through the carboxyl group of PGA initially. This is consistent with the fact, established for the isolated enzyme system by Horecker[16], that the CO_2 is bonded to the number two carbon atom of ribulose diphosphate. More recently Park[19] has shown by means of inhibition studies in broken spinach chloroplasts that ^{14}C entering that system must pass through PGA. That is, PGA is a biochemical intermediate compound—not merely a compound formed by thermal breakdown after the plant is killed.

We shall present here an argument, based on kinetic data, which indicates that the carboxylation of RuDP *in vivo* during photosynthesis gives rise to only one molecule of 3-PGA.

If the ^{14}C which has just entered PGA from $^{14}CO_2$ is subtracted from the total ^{14}C in PGA, the ^{14}C in the remaining carbon atoms of the PGA must all be derived from ribulose diphosphate.

Let us consider the two reactions:



The position of the ^{14}C which has just entered the cycle as $^{14}CO_2$ is indicated by the asterisks. In reaction D, there are five remaining carbon atoms of PGA (numbers 1 to 5) which must be derived from RuDP, while in reaction L there are two such "residual" carbon atoms (numbers 1 and 2). The steady state concentration of PGA in steady state Expt. 18 is 3.0 μmoles of carbon/ml algae, hence the carboxyl carbon concentration is 1.0 μmole of carbon. However, if reaction D is correct, only one-half of this carboxyl carbon, or 0.5 μmole, is derived immediately from CO_2; the other half (carbon atom 3) comes from RuDP. We shall subtract the ^{14}C due to newly incorporated $^{14}CO_2$ from the total ^{14}C found in PGA at each time and for each of these two cases. The specific radioactivity of the remainder may then be compared with the specific radioactivity of the RuDP from which it must be derived.

In order to make this subtraction it is necessary first to calculate the radiocarbon in the carboxyl group of PGA as a function of the time of exposure of the algae to $^{14}CO_2$. This calculation requires in turn a calculation of the saturation curve of the "CO_2 pool", although this could be assumed to be saturated from the beginning without seriously affecting the results.

Consider the steady state system:

$$CO_2 \xrightarrow{R} \text{Pool 1} \xrightarrow{R} \text{Pool 2} \longrightarrow \text{etc.}$$

Let C_1 and C_2 be the steady state concentrations of Pools 1 and 2 and let x and y be the degrees of saturation with ^{14}C of these pools (respectively) as a function of time of exposure of the algae to $^{14}CO_2$. R is the rate of flow of carbon into the system and through the two pools. It is also assumed in this case that the rates of the back reactions are negligible compared to the rates of the forward reactions.

For a small increment of time, the change in degree of saturation is the difference between the rate of flow of ^{14}C into the pool (R) and the rate of flow of carbon out of the pool (Rx), divided by the size of the pool C_1; $\mathrm{d}x/\mathrm{d}t = (R - Rx)/C_1$. Integration and determination of the integration constant at $t = 0$ gives $x = 1 - \text{expt}\ (-R/C_1)t)$.

During a small increment of time, the change in degree of saturation of the second pool is the difference between the rate of flow of ^{14}C into the second pool (Rx) and the rate of flow out (Ry) divided by the pool size C_2;

$$y = \frac{Rx - Ry}{C_2} = \frac{R}{C_2}[1 - \exp(-Rt/C_1) - y]$$

Integration and determination of constants at $t = 0$ leads to two solutions, one for the case $C_1 \neq C_2$:

$$y = 1 - \left(\frac{C_1}{C_1 - C_2}\right)\exp(-Rt/C_1) + \left(\frac{C_2}{C_1 - C_2}\right)\exp(-Rt/C_2)$$

and another for the case $C_1 = C_2$:

$$y = 1 - (1 - Rt/C)\exp(-Rt/C)$$

In applying these equations to the data from steady state Expt. 18 we have assumed a value of $C_1 = 1.2$ μmoles for the "CO_2 pool" (Fig. 1) and a value of 0.2 μmoles/sec (= 12 μmoles/min) for R. The resulting values for x are shown by curve A, Fig. 9.

If reaction D is correct, the PGA carboxyl pool arising from newly incorporated CO_2 is 0.5 μmoles and its degree of saturation y is given by curve B, Fig. 9. If reaction L is correct, this pool is 1.0 μmole and the saturation curve y is that shown as curve C. Curve B times 0.5 and curve C times 1.0 give, as a function of time, the respective μmoles of ^{14}C in the PGA carboxyl pool derived directly from CO_2.

The degree of saturation of the residual carbon atoms of PGA (those which are derived from RuDP) may now be calculated by subtracting from the experimentally determined [^{14}C]PGA these values of the CO_2-derived carboxyl (0.5 B for reaction D, 1.0 C for reaction L) and dividing by the pool sizes of the residual carbons (2.5 and

2.0 respectively). The resulting saturation curves are shown in Fig. 10. In the same figure, Curve R is the saturation curve for ribulose diphosphate, obtained by dividing the experimentally determined ^{14}C labeling of RuDP by its steady state concentration, which was 0.36 μmoles/ml algae.

If the carboxylation of RuDP were to lead to the formation of two molecules of PGA (reaction D), then all of the carbon atoms of RuDP must give rise to the "residual" carbon atoms of PGA. The degree of saturation of these residual carbon atoms at no time could exceed the degree of saturation of the carbon atoms of RuDP. Since the calculated values for these residual atoms, (PGA-0.5 B)/2.5, do exceed those of RuDP at all times after 12 sec, reaction D does not appear to be correct. The curve for reaction L does not exceed the saturation of RuDP until about 1 min. In this case, the residual carbon atoms of PGA are derived only from carbon atoms 2 and 3 of RuDP, and thus may exceed the saturation of the average of carbon atoms 1, 2, 3, 4, and 5 of RuDP. In fact, this is not surprising, since earlier degradation studies on RuDP[1] showed that, during ^{14}C incorporation in photosynthesis, carbon atom 3 is first labeled, followed by carbon atoms 1 and 2, followed finally by carbon atoms 4 and 5. The saturation curve for the residual PGA carbon atoms according to reaction L is thus about as would be expected.

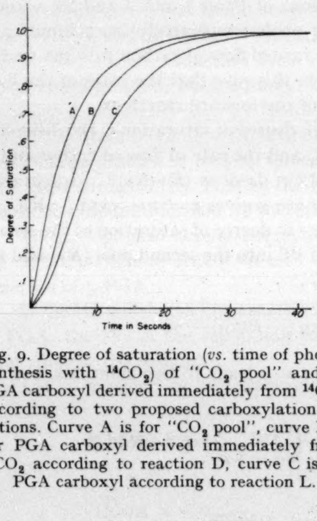


Fig. 9. Degree of saturation (*vs.* time of photosynthesis with $^{14}CO_2$) of "CO_2 pool" and of PGA carboxyl derived immediately from $^{14}CO_2$ according to two proposed carboxylation reactions. Curve A is for "CO_2 pool", curve B is for PGA carboxyl derived immediately from $^{14}CO_2$ according to reaction D, curve C is for PGA carboxyl according to reaction L.

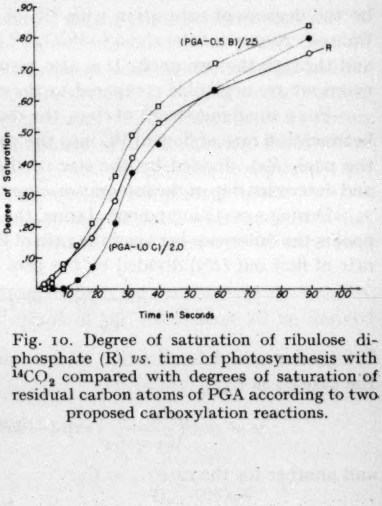


Fig. 10. Degree of saturation of ribulose diphosphate (R) *vs.* time of photosynthesis with $^{14}CO_2$ compared with degrees of saturation of residual carbon atoms of PGA according to two proposed carboxylation reactions.

Note that after 30 sec the carboxyl carbon of PGA would be saturated and the same conclusion could be reached by looking only at the curves from 30 to 90 sec, which are not dependent on the foregoing calculations of CO_2 pool and PGA carboxyl saturation. At these longer times it is sufficient to plot simply the curves for (PGA-0.5)/2.5, (PGA-1.0)/2.0, and RuDP/0.32 all as a function of time.

We conclude, therefore, that the labeling curves for PGA and RuDP in this experiment can best be interpreted as resulting from the occurrence of reaction L. That is, the *in vivo* carboxylation reaction of the carbon reduction cycle during photosynthesis appears to produce one molecule of PGA and one molecule of some other three carbon compound.

Steady state Expt. 28 gave very similar results, from 10 sec to saturation (see Table VI for comparison at 40 sec).

From these experiments alone we cannot identify this three carbon compound. It could be merely a small pool of PGA itself, tightly bound to an enzyme, or in some other way kept apart from the principal PGA pool. Such a pool of PGA molecules, if sufficiently small ($> 0.1\ \mu$mole), would not be distinguishable from the other PGA pool by our methods.

Alternatively, the six carbon product of the carboxylation reaction may be reductively split to one molecule of 3-PGA and one molecule of triose phosphate. In either case, the requirement for the reaction leading to PGA and triose phosphate must be light (or cofactors derived from the light reaction), and the intact chloroplast, or some intact sub-unit of the chloroplast, as it occurs naturally in the living cell.

One cannot say at the present time whether or not any of the chloroplasts or chloroplast fragments isolated from broken cells retain the capacity to carry out such a reductive splitting of the six carbon intermediate of the carbon reduction cycle. In such cell-free systems, the carbon reduction cycle may well operate only via the carboxylation reaction leading to two molecules of free 3-PGA. Recently PARK[20] has prepared electron micrographs of chloroplast and chloroplast fragments which had been found by him to have about as high a rate of photosynthetic CO_2 reduction as any such rates reported for cell-free systems. When compared with electron micrographs of chloroplasts in intact cells, these isolated fragments appear to have undergone considerable physical change, particularly in regard to the apparent density of the stroma and spacing between lamellae. It is possible that the reductive carboxylation pathway, if correct, operates only in the unaltered lamellar system by means of some rather direct transfer of photochemically-produced reducing power from the pigmented layer to the carbon reduction cycle.

If two different three carbon compounds are formed *in vivo* in the light by the carboxylation of RuDP, and if these two products are kept separate until they have been converted to triose phosphate, and react with each other to give hexose, then the resulting hexose molecule might be dissimilarly labeled in its two halves, namely carbon atoms 1, 2, and 3, and carbon atoms 4, 5, and 6. Such asymmetry has been reported by GIBBS AND KANDLER[21,22]. However, other explanations of the phenomenon are also consistent with the carbon reduction cycle[3].

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STUDIES ON A PHOSPHOPROTEIN PHOSPHATASE DERIVED FROM BEEF SPLEEN*

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SUMMARY

1. A modification of the purification procedure of SUNDARARAJAN AND SARMA for beef spleen phosphoprotein phosphatase is described which yields a highly active soluble and stable enzyme preparation.

2. The enzyme cleaves phosphate from nucleotides and from aromatic phosphate esters as well as from phosphoproteins. Other substrates include phosphorylenol pyruvate, phosphoamide, 3-phosphoglyceric acid and fructose-1,6-diphosphate.

3. Enzyme activity is considerably enhanced by reducing agents, ascorbic acid and ferrous ion being the most effective activators. No other metal was found to stimulate the enzyme. Enzyme activity is inhibited by low concentrations of molybdate, 1,10-*o*-phenanthroline, α,α'-dipyridyl and 8-hydroxyquinoline.

4. The enzyme is inactivated by short exposure to reducing agents in the absence of substrates. Ferrous ion protects against this inactivation. A hypothesis to explain these observations is presented.

INTRODUCTION

A study of a phosphoprotein phosphatase derived from beef spleen[2,3] was undertaken in the hope that this enzyme would prove to be a useful tool for the detection and study of various phosphorylated proteins which might be formed as intermediates during oxidative phosphorylation and other phosphate transfer reactions. Since purified preparations of phosphoprotein phosphatase have been shown by SINGER AND FRUTON[4] to cleave phosphoamides and phenyl phosphate as well as phosphoprotein, it was thought desirable to reexamine the substrate specificity of a highly purified preparation particularly in respect to ATP and to characterize its properties before employing the enzyme as a tool in such studies. While the present work was in progress, HOFMAN[5] reported that a protein phosphatase from beef spleen having about one-fifth the specific activity of that of SINGER AND FRUTON cleaved ATP and acetyl phosphate at a rapid rate. More recently the further work of SUNDARARAJAN AND SARMA[6] has confirmed these observations. It is the purpose of this paper to describe the properties of phosphoprotein phosphatase purified from beef spleen by a modification of the procedure of SUNDARARAJAN AND SARMA[2,3], which resulted in

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[*] A preliminary report was presented before the American Society of Biological Chemists[1].

a soluble and stable preparation of high specific activity, and to present additional evidence for the identity of the protein responsible for the cleavage of casein, ATP and several other phosphate esters.

MATERIALS AND METHODS

Substrates

Casein (Hammersten) was obtained from Mann Research Laboratories, Inc., New York. 10 g of casein were dissolved in 85 ml of water with the addition of 16 ml of 0.4 *N* sodium hydroxide and the solution was dialyzed overnight against two changes of 20 volumes of 0.05 *M* acetate buffer of pH 5.8. Phosvitin was a gift of Dr. M. RABINOWITZ. *o*-phosphoserine peptides were kindly supplied by Dr. O. MELLANDER. β-casein phosphopeptone was donated by Dr. R. PETERSON. Glucose-6-phosphate (Ba), glucose-1-phosphate (diK), the nucleoside mono-, di-, and triphosphates, DPN and *p*-nitrophenyl phosphate were products of Sigma Chemical Company, St. Louis, Missouri. DL-o-phosphothreonine, phosphorylenol pyruvate (Ag, Ba), creatine phosphate, bis(*p*-nitrophenyl) phosphate, and *o*-carboxyphenyl phosphate were obtained from the California Foundation for Biochemical Research, Los Angeles, California. β-naphthol phosphate, phenyl phosphate and phosphoamide were generously supplied by Dr. J. CHANLEY. Ribose-5-phosphate, acetyl phosphate and fructose-1,6-diphosphate were products of Schwarz Laboratories, Mt. Vernon, New York. D, L, and DL-o-phosphoserine were kindly given by Dr. M. NEMER. Fructose-1-phosphate and 3-phosphoglyceric acid were products of Boehringer and Soehne, Mannheim, Germany. Cellulose DEAE Type 20 ion exchange agent[7] was a product of the Brown Company, Berlin, New Hampshire.

Analytical procedures

Inorganic phosphate was determined according to LOHMANN AND JENDRASSIK[8], unless otherwise specified. Protein was measured by the quantitative biuret procedure of ROBINSON AND HOGDEN[9] and spectrophotometrically according to WARBURG AND CHRISTIAN[10].

Phosphoprotein phosphatase activity was measured in a test system containing 8 μmoles of casein bound phosphate, 100 μmoles of acetate buffer of pH 5.8, 5 μmoles of neutralized ascorbic acid and enzyme in a total volume of 1 ml. After incubation for 15 or 30 min at 37°, the reaction was stopped by the addition of 1 ml of ice cold 20% trichloroacetic acid and the amount of inorganic phosphate liberated was determined on an aliquot of the supernatant solution after centrifugation. Release of inorganic phosphate from casein was proportional to the concentration of purified enzyme and linear with time under the conditions employed.

Enzyme activity towards ATP was measured in a similar test system in which 5 μmoles of ATP replaced casein as substrate.

A unit of activity is defined as the liberation of 1 μmole of inorganic phosphate/min. Specific activity is expressed as units/mg of enzyme protein as measured by the biuret reaction.

Acid soluble nitrogenous material released when casein was the substrate was measured with Nessler reagent according to KOCH AND MCMEEKIN[11] after digestion with sulfuric acid and hydrogen peroxide.

Enzyme preparation

Modification of the purification procedure of SUNDARARAJAN AND SARMA[2,3] to include removal of nucleic acid by protamine, followed by further purification on a cellulose DEAE column, yielded a soluble enzyme preparation. All operations were carried out at 4° unless otherwise specified.

Step 1. Preparation of crude extract: Six beef spleens collected in ice were freed of capsular material and ground in a meat grinder. Six batches of 400 g of minced tissue were homogenized with 1 l of a 0.5 *M* sodium chloride–0.2 *M* acetate solution of pH 5.0 in a large Waring blendor with a capacity of 7.5 l and overhead drive. The homogenate was centrifuged immediately at 1,000 × *g* for 15 min and the supernatant solution was filtered through several layers of gauze (4,600 ml).

Step 2. Ammonium sulfate fractionation: 22.6 g of solid ammonium sulfate/100 ml of crude extract were added and after stirring for 30 min the mixture was centrifuged at 16,000 × *g* for 20 min. The precipitate was discarded and 26 g of solid ammonium sulfate/100 ml of supernatant solution were added. After 30 min the mixture was centrifuged as before and the precipitate was taken up in a minimum volume of water (about 300 ml). Dialysis with stirring against 8 l of glass distilled water (5 to 6 changes) was continued (about 48 h) until the dialysate was free of sulfate ion. The precipitate formed during dialysis contained the major portion of the enzyme and was separated from the supernatant solution by centrifugation. The precipitate was extracted twice with 0.5 *M* sodium chloride–0.2 *M* acetate solution of pH 5.0, spinning after each extraction at 100,000 × *g* for 45 min in a Spinco centrifuge. The ratio of the u.v. extinction of the combined extracts (178 ml) at 280 mμ and 260 mμ was 0.65–0.72 indicating the presence of about 11 to 14% nucleic acid.

Step 3. Heat and protamine sulfate treatment: The reddish solution was divided into 15-ml aliquots and heated for 5 min in test tubes in a water bath maintained at 70°. The solutions were then quickly chilled in an ice bath and centrifuged to remove denatured protein. The clear amber supernatant solution contained over 90 % of the total activity of the unheated enzyme, and about 25% of the protein. However, the ratio of u.v. extinction at 280 mμ and 260 mμ did not change significantly. Attempts at further purification of this preparation on a DEAE cellulose column were not reproducible because of the presence of the nucleic acid and several peaks of activity emerged on fractional elution. Consequently, the enzyme solution was treated with protamine sulfate. To 164 ml of the heated enzyme, 15 ml of a 2% protamine sulfate solution were added at room temperature and after 10 min the mixture was centrifuged at 8,000 × *g* for 15 min. The enzyme was precipitated from the supernatant solution, which retained all the activity, by the addition of 56 g of solid ammonium sulfate/100 ml. After centrifugation the enzyme was taken up in about 8 ml of water and dialyzed overnight against 3 l of 0.005 *M* sodium chloride–0.002 *M* acetate solution of pH 6.0. A precipitate which formed contained no activity and was discarded. The clear yellow-brown supernatant solution (17.8 ml) had a ratio of u.v. extinction at 280 mμ and 260 mμ of 1.35.

Step 4. Fractionation on a cellulose DEAE column: The enzyme solution was applied to a DEAE cellulose anion exchange column (5 × 3.5 cm), which had been equilibrated with 0.005 *M* sodium chloride–0.002 *M* acetate solution of pH 6.0. 10-ml fractions were collected. In contrast to the nucleoprotein preparation before protamine treatment, the enzyme did not adhere to the cellulose and was eluted in tubes 4, 5 and 6 upon washing with the equilibrating buffer solution. The ratio of u.v. extinction at 280 mμ and 260 mμ of the purified enzyme at this stage was 1.45.

RESULTS

Specificity of the purified enzyme

A summary of a typical purification of the enzyme is presented in Table I. Activity towards ATP, a common substrate in phosphate transfer reactions, as well as towards the phosphoprotein casein, was followed throughout the purification procedure. It can be seen that the ratio of ATPase to casein phosphatase activity remained constant throughout the latter steps of the preparation, suggesting that these two activities reside in the same protein. At a concentration of 2 μmoles/ml casein was completely dephosphorylated by 25 μg of the purified spleen enzyme in 1 h without the release of significant amounts of acid soluble nitrogenous material (less than 0.05 mole of nitrogen/mole of phosphate released). When ATP was the substrate the two terminal phosphates were liberated.

A survey of the substrate specificity of the purified enzyme preparation is summarized in Table II. In confirmation of previous reports[4–6] the enzyme attacked most rapidly the two terminal phosphates of ATP and other nucleotides, as well as aromatic phosphate esters. Inorganic pyrophosphate and phosphoproteins were hydrolyzed at about 1/6 the rate of ATP. Phosphorylenol pyruvate was found to be an excellent substrate which was split about twice as fast as casein. The rapid cleavage of acetyl phosphate has been reported previously[5]. Phosphoamide was hydrolyzed at about 0.5 the rate of casein. Others[4] have observed a somewhat more rapid hydrolysis of this substrate, but experimental conditions were not strictly comparable. In particular, creatine phosphate was cleaved only sluggishly with the enzyme preparation described in the present paper. Some aliphatic phosphate esters, such as fructose-1,6-diphosphate, adenosine-5′-phosphate and 3-phosphoglyceric acid, were cleaved more rapidly than creatine phosphate. The observed hydrolysis of 3-phosphoglyceric acid at 0.1 the rate of casein is contrary to previous reports and will be discussed later. Among the substrates attacked comparatively slowly are phosphoserine and phosphothreonine, which were hydrolyzed at about 1 % the rate of casein. Phosphoserine peptides were hydrolyzed somewhat faster, the rate apparently increasing with the chain lengths of the peptides.

Activation of enzyme by reducing agents

In agreement with the observations of previous investigators[2,4], it was found that enzyme activity was considerably enhanced by the presence of reducing agents. As various compounds have been employed, it was of interest to compare their stimulation of the casein phosphatase activity. It can be seen in Fig. 1 that ascorbic acid and thioglycollic acid were the best activators, glutathione was barely active, while α-thioglycerol, cysteine and 2-mercaptoethanol were intermediate in their effectiveness. Experiments with another enzyme preparation showed a somewhat lower activation by cysteine and 2-mercaptoethanol, ascorbic acid yielding maximal results and glutathione being ineffective. With ATP as substrate ascorbic acid was also most effective, while thioglycollic acid was only about half as active at the optimal concen-

TABLE I

PURIFICATION OF PHOSPHOPROTEIN PHOSPHATASE

Step	*Volume ml*	*Protein g*	*Activity (Casein) Units*	*Specific activity (Casein) Units/mg protein*	*Activity (ATP) Units*	*Specific activity (ATP) Units/mg protein*	*ATP/Casein*
1	4,600	165	3,550	0.022	26,700	0.16	7.3
2	178	2.3	1,000	0.44	6,200	2.7	6.1
3	17.8	0.178	600	3.4	3,570	20.0	5.9
4	33	0.096	600	6.3	3,620	38.0	6.0

TABLE II

SPECIFICITY OF PHOSPHOPROTEIN PHOSPHATASE

Test system: 5 µmoles of substrate, 100 µmoles of acetate buffer of pH 5.8, 5 µmoles of neutralized ascorbic acid and 10 µg of enzyme (specific activity with casein = 6.0) in a final volume of 1 ml incubated at 37° for 15 or 30 min. Meaning of the number in brackets: (1) Phosphate release from pyrophosphate was divided by 2, since two moles of inorganic phosphate are released for each mole of substrate cleaved. (2) Measured by the appearance of *p*-nitrophenol[12] and of inorganic phosphate. (3) Disappearance of DPN was measured spectrophotometrically with alcohol dehydrogenase. (4) Measured by a modification of the LOWRY-LOPEZ procedure[13, 14] with color development at ice bath temperature to reduce the rate of non-enzymic hydrolysis[15]. (5) Measured as the change in creatine phosphate concentration (acid-labile phosphate) after removal of inorganic phosphate by $CaCl_2$ precipitation[16, 17]. (6) Measured as the change in acetyl phosphate concentration by the hydroxamic acid procedure of LIPMANN AND TUTTLE[18].

Substrate	*µg phosphate liberated*	
	15 min	*30 min*
Phosphoproteins		
Casein	26	48
Phosvitin		39
Phosphopeptides		
Ser–P·gly		0.4
Gly·ser–P		0.8
Gly·ser–P·gly		2.4
β-casein phosphopeptone	4	
Phosphomonoesters		
Glucose-6-phosphate		2.1
Glucose-1-phosphate		0
Fructose-6-phosphate		1.3
Fructose-1-phosphate		1.8
Fructose-1,6-diphosphate		8.6
Ribose-5-phosphate		2.9
Ribulose-1,5-diphosphate	9.3	
Adenosine-5′-phosphate		11.0
α- or *β*-glycerol phosphate		0.5
3-phosphoglyceric acid		5.4
Phosphoserine (D, L, DL)		0.5
Phosphothreonine (DL)		0.6
β-naphthol phosphate	69**	
p-nitrophenyl phosphate	34** (2)	
o-carboxyphenyl phosphate	50**	
Phenyl phosphate	41**	
Pyrophosphates		
ATP	78**	
CTP*	52**	
GTP*	19.5**	
ADP	78**	
UDP*	78**	
Inorganic pyrophosphate	25 (1)	
Phosphodiesters		
Bis(*p*-nitrophenyl) phosphate		1.2 (2)
DPN		0 (3)
Phosphoamides		
Phosphoamide		23 (4)
Creatine phosphate		1.5 (5)
Acyl phosphate		
Acetyl phosphate	26 (6)	
Enol phosphate		
Phosphorylenol phosphate	50	

* 3.3 mg of substrate in 1 ml.
** 5 µg of enzyme used.

tration of $10^{-2}\,M$. In view of these data, ascorbic acid ($5 \cdot 10^{-3}\,M$) was selected for routine assay procedure.

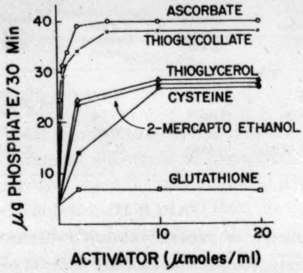

Fig. 1. Activation of casein phosphatase by reducing agents. Each test tube contained in a final volume of 1 ml, 100 μmoles of acetate buffer of pH 5.8, 8 μmoles of casein phosphate, 8 μg of enzyme (specific activity with casein = 6.0) and reducing agent as indicated. After 30 min incubation at 37°, the reactions were stopped by the addition of 1 ml of cold 20% trichloroacetic acid and the samples were analyzed for inorganic phosphate.

Effect of metals

The reducing agents could be completely replaced by ferrous ion, which was as effective as ascorbic acid, giving maximal stimulation at $10^{-3}\,M$ with casein and at $4 \cdot 10^{-4}\,M$ with ATP as substrate. The difference in concentration of metal ion required for optimal activity with these two substrates may be simply a reflection of the binding capacity of casein for iron[19], thus reducing the effective concentration available to the enzyme. Higher concentrations of ferrous ion showed less stimulation and actually inhibited enzyme activity at a concentration of $5 \cdot 10^{-3}\,M$.

Metals commonly associated with phosphate transfer reactions: calcium, magnesium and manganese, did not stimulate enzyme activity with either casein or ATP when tested in the absence of reducing agents and became inhibitory at about $10^{-2}\,M$. Cu^{++}, Co^{++}, Ni^{++}, Zn^{++} or Fe^{++} did not stimulate either and in fact caused about 50% inhibition at about $10^{-3}\,M$.

Effect of inhibitors

In line with this suggestive evidence of a metal participant, various chelating agents were found to inhibit enzyme activity. Table III summarizes the effect of various inhibitors with ATP and casein as substrates. Molybdate was the most effective agent, giving 50% inhibition at about $4 \cdot 10^{-6}\,M$ and complete inhibition at $10^{-4}\,M$. 1,10-*o*-phenanthroline; *a,a'*-dipyridyl and 8-hydroxyquinoline, which are particularly effective in sequestering iron[20], were all effective inhibitors. With the exception of fluoride the extent of inhibition by each compound was the same with both substrates.

p-chloromercuribenzoate, even at high concentration ($10^{-3}\,M$), inhibited ATPase only by 40%. Casein phosphatase activity was even less susceptible and only if the enzyme was preincubated for 15 min with the inhibitor before the addition of the substrate, suggesting that casein may bind this inhibitor. Also, high concentrations of iodoacetate ($10^{-2}\,M$) gave only 30% inhibition of the casein phosphatase activity with or without preincubation.

Phosphate, a reaction product, gave 40 % inhibition of casein phosphatase activity at 10^{-2} M. Azide, cyanide and ethylenediamine tetraacetate were without effect below 10^{-2} M on the ascorbic acid activated enzyme with either ATP or casein as substrate.

TABLE III

EFFECT OF INHIBITORS ON CASEIN PHOSPHATASE AND ON ATPASE ACTIVITY

Each test tube contained in a final volume of 1 ml, 100 µmoles of acetate buffer of pH 5.8, 5 µmoles of neutralized ascorbic acid, either 5 µmoles of ATP or 8 µmoles of casein phosphate, inhibitor as indicated and 10 µg of enzyme (specific activity with casein = 5.0). After incubation at 37° for 15 min, the reactions were stopped by the addition of 1 ml of 20 % trichloroacetic acid and the samples were analyzed for inorganic phosphate. Data are expressed as % inhibition of phosphate release.

Inhibitor M	*Phenanthroline*		*α,α′-dipyridyl*		*8-hydroxyquinolin*		*Molybdate*		*Fluoride*	
	*Casein**	*ATP*	*Casein**	*ATP*	*Casein**	*ATP*	*Casein*	*ATP*	*Casein*	*ATP*
10^{-7}	—	—	—	—	—	—	0	0	—	—
10^{-6}	0	0	0	0	0	0	25	30	—	—
10^{-5}	55	58	0	0	0	0	76	72	—	—
$4 \cdot 10^{-5}$	68	71	54	47	0	10	—	—	—	—
10^{-4}	68	71	75	62	25	30	97	92	—	—
$4 \cdot 10^{-4}$	69	71	79	74	40	45	—	—	—	—
10^{-3}	70	71	84	73	49	42	100	100	18	58
$4 \cdot 10^{-3}$	—	—	—	—	—	—	—	—	52	84
10^{-2}	—	—	—	—	—	—	—	—	63	89

* Casein was dialyzed first against 100 volumes of 0.002 M ethylenediamine tetraacetate and then against 100 volumes of 0.05 M acetate buffer of pH 5.8 before use.

Inactivation of enzyme activity by reducing agents

It was observed that temperature equilibration of enzyme with buffer and reducing agent for variable time intervals before starting the reaction by the addition of substrate resulted in variable activity. Investigation of this phenomenon revealed that the activating agents ascorbic acid and thioglycollic acid, but not ferrous ion, brought about the irreversible inactivation of the enzyme in the absence of substrate upon incubation at 37°. The time course of inactivation by ascorbic acid and by thioglycollic acid of the casein phosphatase and the ATPase activities of the enzyme is shown in Fig. 2. Within 20 min there was about a 90 % loss of activity in the presence of ascorbic acid. Enzyme in the presence of buffer alone was stable under these conditions. Addition of ferrous ion or more reducing agent or both did not reactivate the enzyme. Addition of fresh enzyme, however, gave full activity proportional to the amount of enzyme added to the mixture. The hydrolysis of all active substrates was abolished to the same extent by this inactivation procedure.

Ferrous ion protection against the ascorbic acid inactivation of the enzyme (Fig. 3)

Ferrous ions, unlike other activators of enzyme activity, did not cause the inactivation of the enzyme during a 20 min preincubation period in the absence of substrate. In fact, ferrous ions, over the range of 10^{-5} to 10^{-3} M, effectively protected against the inactivation by ascorbic acid. None of the other metal ions previously listed, with the exception of ferric ion, which is presumably reduced to ferrous ion by ascorbic acid, protected the enzyme.

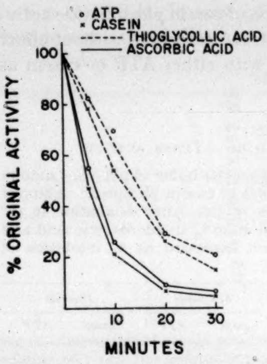

Fig. 2. Enzyme inactivation by reducing agents in the absence of substrate. Each test tube contained in a volume of 0.5 ml, 100 μmoles of acetate buffer of pH 5.8, 5 μmoles of neutralized ascorbic acid or thioglycollic acid and 10 μg of enzyme (specific activity with casein = 5.0). After preincubation at 37° for the time intervals indicated, 0.5 ml containing either 5 μmoles of ATP or 8 μmoles of casein phosphate was added and the incubation continued for 15 min. The reactions were stopped by the addition of 1 ml of cold 20% trichloroacetic acid and the samples were analyzed for inorganic phosphate.

Fig. 3. Ferrous ion protection against ascorbic acid inactivation of casein phosphatase activity. Experimental test tubes contained in a volume of 0.5 ml, 100 μmoles of acetate buffer of pH 5.8, 10 μg of enzyme (specific activity with casein = 5.0) and, as indicated, 5 μmoles of neutralized ascorbic acid and/or 0.5 μmoles of ferrous chloride. After preincubation at 37° for the time intervals indicated, 0.5 ml containing 8 μmoles of casein phosphate was added and incubation continued for 15 min. 5 μmoles of neutralized ascorbic acid were added to the vessels containing enzyme alone for the assay of enzyme activity during the second incubation period. The reactions were stopped by the addition of 1 ml of cold 20% trichloroacetic acid and the samples were analyzed for inorganic phosphate.

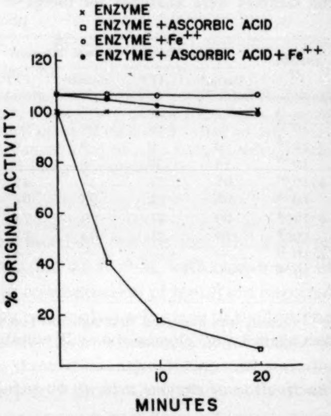

TABLE IV

EFFECT OF PREINCUBATION UNDER VARIOUS CONDITIONS ON ENZYME ACTIVITY WITH SEVERAL SUBSTRATES

Experimental test tubes with no preincubation contained in a final volume of 1 ml, 100 μmoles of acetate buffer of pH 5.8, 5 μmoles of substrate, 8 μg of enzyme (specific activity with casein = 6.0) and, where indicated, 5 μmoles of neutralized ascorbic acid and 0.5 μmoles of ferrous chloride. Incubation was at 37° for 15 min. Experimental test tubes which were preincubated contained in a volume of 0.5 ml, 100 μmoles of acetate buffer of pH 5.8, 8 μg of enzyme and, where indicated, 5 μmoles of neutralized ascorbic acid and 0.5 μmoles of ferrous chloride. After 20 min preincubation at 37°, 5 μmoles of substrate in a volume of 0.5 ml were added to each test tube and incubation was continued for 15 min. All reactions were stopped by the addition of 1 ml of cold 20% trichloroacetic acid and analysis for inorganic phosphate was carried out as described in METHODS. Results are expressed as μg of phosphate liberated in 15 min.

Substrate	*No preincubation*			*Preincubation*		
	1 *No reducing agent*	*2* *Ascorbic acid*	*3* *Ascorbic acid + ferrous chloride*	*4* *No reducing agent**	*5* *Ascorbic acid*	*6* *Ascorbic acid + ferrous chloride*
Casein	1.4	21	23	20	0.5	19
ATP	19	125	133	130	11	125
Pyrophosphate**	4.0	20	43	27	1.0	35
Phenylphosphate	13	55	102	80	5.5	95

* Ascorbic acid added after preincubation period for assay of enzyme activity.
** Measured as described in Table II.

Table IV shows the effect of preincubation of enzyme under various conditions on the activity with ATP, pyrophosphate, phenyl phosphate and casein as substrate. Stimulation by ascorbic acid is shown in column 2, a slight further stimulation by Fe^{++} is shown in column 3. Stability of activity on incubation at 37° without ascorbic acid is shown in column 4. Inactivation by ascorbic acid in the absence of substrate is shown in column 5 and protection by ferrous ions against this inactivation is shown in column 6.

Experiments with phosphoproteins

Although the wide range of specificity of the purified spleen enzyme reduces its usefulness as an analytical tool to study phosphoprotein intermediates in various enzyme reactions, some attempts were made to explore the feasibility of this approach. Phosphoglucomutase isolated from rabbit muscle is a phosphoprotein and does not react with glucose-1,6-diphosphate without a suitable phosphate acceptor[21, 22]. Action of the phosphoprotein phosphatase on this phosphoprotein was determined by measuring its reactivity with glucose-1,6-diphosphate before and after treatment with the phosphatase. Following incubation of phosphoglucomutase (0.05 μmole) with 50 μg of the purified phosphoprotein phosphatase, addition of glucose-1,6-diphosphate resulted in the formation of glucose-6-phosphate in amounts stoichiometric with phosphoglucomutase. However, a control test tube which contained only the phosphatase activator, ascorbic acid, yielded the same amount of dephosphoglucomutase. Phosphoglucomutase incubated alone in a second control test tube did not accept a phosphate group from glucose-1,6-diphosphate. The interesting possibility that reducing agents, which are known to activate phosphoglucomutase, may labilize the phosphoenzyme was not pursued further. Although a kinetic analysis may have revealed an effect of the phosphatase in addition to ascorbic acid this approach was difficult with such a scarce substrate.

Twice recrystallized hexokinase prepared according to Darrow and Colowick* was found to contain organically bound phosphate (about 1.5 moles/90,000 g). However, spleen phosphoprotein phosphatase did not release this phosphate group. In any case, preliminary experiments on phosphate transfer with hexokinase failed to reveal the formation of a phosphoprotein. A more direct approach to enzymes which are assumed to form a phosphoenzyme in the presence of ATP, by transforming them to ATPases in the presence of a specific phosphoprotein phosphatase was precluded by the rapid cleavage of ATP by the spleen enzyme.

DISCUSSION

The primary purpose of this investigation was to prepare a phosphoprotein phosphatase that could catalyze the dephosphorylation of phosphoenzymes which are assumed to be formed during oxidative phosphorylation and during some other phosphate transfer reactions. This aim was not fulfilled because a separation of the phosphoprotein phosphatase activity from enzyme activity towards ATP and other phosphate esters which participate in phosphate transfer reactions has not been achieved. In fact, further evidence has been obtained that the hydrolysis of ATP and other phosphate esters is catalyzed by the same protein as has been suggested previously[1-6]. Several additional compounds such as phosphorylenol pyruvate, 3-phosphoglyceric acid and fructose-1,6-diphosphate, have been added to the list of hydrolyzable substrates. The relatively rapid cleavage of 3-phosphoglyceric acid as compared with α or β glycerol phosphate points to a large influence of neighboring groups on the susceptibility of the substrate to the enzyme.

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* Unpublished preparation of crystalline yeast hexokinase. We wish to thank Dr. Colowick for this information.

In view of these considerations it is not possible to draw any significant conclusions concerning the nature of the phosphate linkage in casein from the fact that all the phosphate groups are cleaved by this enzyme. Nor does the fact that phosphoserine and phosphothreonine are hydrolyzed only slowly eliminate these amino acids as bearers of the phosphate groups in casein, since neighboring peptide linkages and a specific amino acid sequence may profoundly influence susceptibility to hydrolysis[23].

The purification procedure described in this paper results in a highly active soluble phosphoprotein phosphatase of considerable stability. Difficulties in reproducing the original procedure of SUNDARARAJAN AND SARMA[2, 3] were traced to the presence of nucleic acids which render the enzyme less soluble. In the course of purification of such preparations smearing of activity was observed, particularly with DEAE cellulose columns from which the nucleoproteins emerged in several rather distinct peaks bearing phosphoprotein phosphatase and ATPase activity of constant ratios. In contrast to the nucleoprotein, the soluble enzyme obtained after treatment with protamine sulfate was not retained by the DEAE cellulose, but was readily absorbed on a cation exchange resin such as Dowex-50. These properties suggest that the free enzyme is a strongly basic protein.

The method of purification described by SINGER AND FRUTON[1] is somewhat simpler than the present one and results in a preparation of approximately the same specific activity. Although for many purposes their method is probably preferable, it should be pointed out that the preparations seemed to differ in several minor aspects and one very critical one. While the perhaps more gentle extraction with salts used in the present work results in an enzyme which is rather inactive in the absence of reducing agents and which is fully activated by ferrous ions, the enzyme preparation of SINGER AND FRUTON obtained by extraction of the spleen at pH 3.0 for 22 h at room temperature is less dependent on reducing agents and is inhibited by ferrous ions. This rather puzzling feature is somewhat reminiscent of the observations of MOKRASCH AND MCGILVARY[24,25] with fructose diphosphatase from liver. Prolonged autolysis at an acid pH, or exposure to proteolytic enzymes, not only increased the activity, but led to alterations in the physical properties of the enzyme. BYRNE[26] also observed that fructose diphosphatase, obtained by mild procedures, cleaves fructose diphosphate at neutrality, in contrast to the enzyme obtained by autolysis at a low pH. It is conceivable that some of the differences observed among protein phosphatases from spleen, such as in the reactivity with creatinephosphate, are due to similar alterations in pH optimum.

Among the minor differences in properties of the protein phosphatase prepared by the two procedures is the cleavage of aliphatic esters, such as 3-phosphoglyceric acid. The hydrolysis of this compound, as well as that of ribulose-1,5-diphosphate and fructose-1,6-diphosphate, in contrast to the much lower reactivity of α or β glycerol phosphate, ribose-5-phosphate, fructose-1-phosphate and fructose-6-phosphate, may be related to the presence of a second acidic group in the more susceptible substrates. The possibility that these aliphatic phosphate esters are cleaved by a contaminating non-specific phosphatase has not been rigidly ruled out, but it is unlikely because, after inactivation of the phosphoprotein phosphatase activity by short exposure to reducing agents in the absence of substrate, the hydrolysis of these simple esters is also eliminated.

As has been previously observed[2,4], sulfhydryl compounds enhance considerably the activity of beef spleen phosphoprotein phosphatase. With the present preparation the most effective activator was ascorbic acid and, in contrast to other reports, ferrous ions were found to be equally effective. No other metal ion tested stimulated enzyme activity. PAIGEN[19] recently reported the activation of a protein phosphatase obtained from mouse liver by Cu^{++}, Fe^{++} and Ni^{++}. However, the activity was both sulfhydryl and metal dependent, while the spleen enzyme is fully active with ferrous ion alone in the absence of added reducing agents. The concentration of ascorbic acid used in these experiments is not likely to effect the reduction of an S–S group on the enzyme. These observations, together with the relative resistance of enzymic activity to iodoacetate and to *p*-chloromercuribenzoate, cast doubts on the validity of proposed mechanisms of an active site involving an SH group. The inhibition of enzyme activity by metal chelating agents such as 1,10-*o*-phenanthroline, α,α'-dipyridyl and 8-hydroxyquinoline, strongly points to the participation of a metal, probably ferrous ion.

The stability properties of the spleen protein phosphatase are rather unusual. While the activity is preserved after exposure to temperatures up to 70° for prolonged time periods[3,4] the enzyme is rapidly inactivated, even at 37°, in the presence of ascorbic acid or thioglycollic acid. Ferrous ions as well as substrate serve to protect the enzyme against inactivation in the presence of reducing agents. The following hypothesis serves to explain the above findings. The enzyme is isolated from the spleen as a ferric-enzyme complex which has a very low dissociation constant and is of considerable stability to heat. For activity, it is visualized that the ferric-enzyme has to be reduced to form a ferrous-enzyme complex which is more readily dissociable. This ferrous-enzyme complex is stabilized in the presence of substrate. Without substrate the complex dissociates and the metal-less enzyme is rapidly inactivated. Addition of ferrous ions at relatively high concentrations protects the enzyme by simply providing more favorable equilibrium conditions for the maintenance of a ferrous-enzyme complex.

While this manuscript was in preparation, two additional reports on the purification of beef spleen phosphoprotein phosphatase with specific activities two to eight times higher than the present one have been described[6,27]. However, these workers were not concerned with the specific problems of metal activation and substrate specificity dealt with in the present communication.

ACKNOWLEDGEMENTS

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THE BIOSYNTHESIS OF CHOLINE AND ITS RELATION TO PHOSPHOLIPID METABOLISM

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SUMMARY

The biosynthesis of choline has been studied in the rat with [3-^{14}C]serine, [1,2-^{14}C]-aminoethanol and [$^{14}CH_3$]methionine as precursors.

When [^{14}C]aminoethanol was given to intact rats, radioactive choline was isolated from the liver phospholipids, but no radioactive phosphocholine was found in an aqueous liver extract. When [^{14}C]methionine was given, radioactive mono- and dimethylaminoethanol, as well as radioactive choline, were isolated from the liver phospholipids. The mono- and dimethylaminoethanol had much higher turnover rates than had the choline. When [^{14}C]methionine was administered, the incorporation of radioactivity into cytidinediphosphocholine was, in comparison with the incorporation of radioactivity into phospholipid choline, delayed.

In vitro experiments showed that choline-labeled lecithin was formed when isolated rat-liver microsomes were incubated with S-adenosylmethionine.

When [^{14}C]serine was injected into intact rats, radioactive aminoethanol as well as radioactive choline labeled in both the hydroxyethyl- and in the methyl groups, were isolated. Turnover and specific activity studies showed that phosphoaminoethanol and cytidinediphosphoaminoethanol are unlikely intermediates in the formation of phospholipid aminoethanol from serine.

On the basis of these results it is concluded that phosphatidylserine is decarboxylated to phosphatidylaminoethanol and that the latter compound is methylated to form lecithin. In this reaction all the methyl groups of choline are incorporated by transmethylation from S-adenosylmethionine.

The significance of these results is discussed in relation to fat and phospholipid metabolism.

INTRODUCTION

The decarboxylation of serine to aminoethanol and the subsequent methylation of aminoethanol to choline are well known reactions[1,2]. The β-carbon atom of serine is also known to be a precursor of the methyl groups of methionine and choline, presumably through the intermediate formation of a tetrahydrofolic acid–formaldehyde complex. The methyl group of methionine is known to be transferred to choline as an intact unit[3,4]. On the basis of *in vivo* experiments, STEKOL *et al.*[5] have suggested that the first two methyl groups of choline are synthesized *de novo* and incorporated into choline by a folic acid-dependent mechanism, while the third methyl group is incorporated by transmethylation from adenosylmethionine[4].

We have recently performed extensive *in vitro* experiments to elucidate the mechanism involved in the biosynthesis of choline from free aminoethanol or [^{14}C]-phosphoaminoethanol, but no free choline formation could be detected in liver homogenates. These negative results might indicate that a derivative of aminoethanol rather than the free compound is the methyl acceptor in the biosynthesis of choline.

The following reactions are known for the conversion of the above bases to phosphatides[6].

Aminoethanol ⟶ phosphoaminoethanol
⟶ cytidinediphosphoaminoethanol ⟶ phosphatidylaminoethanol (1)

Choline ⟶ phosphocholine ⟶ cytidinediphosphocholine ⟶ phosphatidylcholine (2)

It is thus evident that there are at least four different possible methyl acceptors in the biosynthesis of choline.

In the conversion of serine to phosphatidylserine, no intermediates are known[7], but phosphoserine and "cytidinediphosphoserine" must be considered as possible substrates for a "serine decarboxylase".

On the basis of these considerations, experiments were designed to identify the immediate precursors in the methylation of aminoethanol and also in the decarboxylation of serine. There are measurable pools in rat liver of phosphoaminoethanol[8], phosphocholine[9], cytidinediphosphoaminoethanol and cytidinediphosphocholine[6]. By injecting the known precursors, [^{14}C]serine, [^{14}C]aminoethanol, [^{14}C]choline and [^{14}C]methionine, and following the appearance of label in the phospholipids and in the above mentioned phospholipid precursors, we have found it possible to exclude certain of the possible pathways.

In this paper results are reported which indicate that it is the fully formed phosphatidylserine which is first decarboxylated, and subsequently methylated, to lecithin.

Preliminary reports on some of these results have already been published[10, 11].

MATERIALS

The ^{14}C precursors used were all purchased from Isotopes Specialities Co., Burbank. California. The [3-^{14}C]serine had a specific activity of ~ 2.1 mC/mmole, the [1,2-^{14}C]-aminoethanol* ~ 1 mC/mmole, the [^{14}CH$_3$]choline ~ 1 mC/mmole and the [^{14}CH$_3$]-methionine ~ 4.5 mC/mmole. When lower specific activities were sufficient, the labeled compounds were diluted with corresponding unlabeled material before use.

The *Clostridium welchii* toxin (200–400 MLD/ml) was kindly supplied by Lederle Laboratories, Pearl River, N.Y.

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* We are indebted to Dr. M. ROTHSTEIN of this laboratory for preparing [1,2-^{14}C]phosphoaminoethanol by the following procedure: Aminoethanol hydrochloride (0.3 mmole) in 100% phosphoric acid was kept at 130° for 24 h. After removal of the phosphate with barium hydroxide, the phosphoaminoethanol was eluted from a Dowex 50 (H$^+$) column with 1 *N* acetic acid. The product contained no detectable impurities on paper chromatographs.

METHODS

Female rats weighing 250–300 g were used for *in vivo* experiments. Both sexes were used for *in vitro* experiments. The animals had free access to food and water until they were sacrificed. All radioactive compounds were administered intraperitoneally in 1–2 ml of 0.9 % NaCl. *In vitro* techniques were as reported elsewhere[11].

Preparation of aqueous liver extracts and isolation of liver phospholipids

The method used by DAWSON[12] was modified for the work with whole rat livers. The animals were sacrificed by decapitation and bled as thoroughly as possible. The livers were immediately homogenized in 80–90 ml of 67 % ethanol in a Waring blendor. After centrifugation, the homogenate residues were extracted three more times with 25–30 ml of 67 % ethanol and the combined extracts were concentrated to approximately half the original volume, cleared by centrifugation and then shaken twice with chloroform (50 and 30 ml). The phases were separated by centrifugation and the clear water phase was removed by pipette for column chromatography.

The chloroform–ethanol layers were used to complete the lipid extraction from the homogenate residue. An additional extraction was done with pure chloroform. The chloroform–ethanol was heated to boiling at each extraction.

The combined extracts were evaporated to dryness and the residues extracted with petroleum ether (b.p. 30–60°). The petroleum ether extracts were washed with a little water and concentrated to 3–4 ml and the phospholipids were precipitated by the addition of 8–10 volumes of acetone at —10°. The acetone precipitation was repeated once.

Hydrolysis of phospholipids

The isolated phospholipids were hydrolyzed in 1.2 *N* HCl, according to the procedure of PILGERAM *et al.*[2]. After removal of the fatty acids by filtration the mixture was evaporated to dryness and the residue was dissolved in a minimal volume of water for column chromatography.

Column chromatography

The phospholipid bases were separated chromatographically according to PILGERAM *et al.*[2]. This procedure was also found to give separate peaks for the mono- and dimethylaminoethanol (Fig. 1).

Phosphoaminoethanol and phosphocholine were isolated by chromatographing the aqueous liver extracts on the same type of column as the free phospholipid bases. The phosphoderivatives of aminoethanol, mono- and dimethylaminoethanol and choline were eluted in the order mentioned by approx. 7, 10, 13 and 18 resin bed volumes of 2 *N* formic acid.

Cytidinediphosphoaminoethanol and -choline were obtained in separate chromatographic peaks by KENNEDY's procedure[13], employing a Dowex-1-formate column when 0.02 *N* formic acid was used in the upper reservoir instead of 0.04 *N* acid. Fractions of 5 ml were collected. Fig. 2 shows two typical runs.

Separation of phospholipids was performed according to the procedure of MARINETTI *et al.*[14]. The silicic acid column gave good separation of the "cephalin" and lecithin components of the mixture (Fig. 5A).

Verification of identity of isolated radioactive compounds: In addition to the elution points in the column chromatograms, the identity of each of the compounds was verified by paper chromatography.

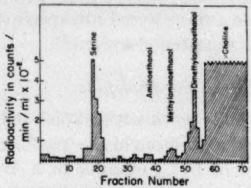

Fig. 1. Radioactivity distribution in column chromatogram of rat-liver phospholipid bases isolated from rat injected with approximately 11.5 μmoles [$^{14}CH_3$]methionine, 3.9·10^6 counts/min. Animal sacrificed 40 min after the injection; 5 ml fractions collected from the column.

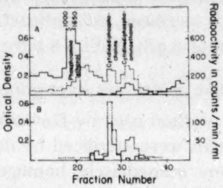

Fig. 2. Radioactivity distribution and u.v. light absorption at 280 mμ in column chromatogram of rat liver aqueous extract from rats injected with: A: approximately 100 μmoles [1,2-^{14}C]-aminoethanol, 6·10^6 counts/min. The animal was sacrificed 75 min after the injection. B: approx. 6 μmoles [$^{14}CH_3$]methionine, 1.9·10^6 counts/min. The animal was sacrificed 300 min after the injection. Fractions of approx. 5 ml collected.

Phospholipid bases

The isolated serine gave the same R_F value as the authentic compound on paper chromatograms developed with *n*-butanol–diethyleneglycol–water (4:1:1) (see ref. 15). Whatman No. 4 filter paper was used in this and all other paper chromatography systems.

The identities of aminoethanol, mono- and dimethylaminoethanol and choline were established by the paper chromatography system described below. Fifty g phenol, 50 ml *n*-butanol, 3 ml 80% formic acid and 5 ml water were shaken with solid potassium chloride. The paper was pre-treated with 1 *N* potassium chloride and dried. The salt treatment of the system reduced tailing in the chromatograms. In this system the following R_F values were found: aminoethanol, 0.10; methylaminoethanol, 0.30; dimethylaminoethanol, 0.55 and choline, 0.72. The compounds isolated from rat liver phospholipids gave identical R_F values, estimated from the distribution of radioactivity on the chromatograms. Aminoethanol gave a positive ninhydrin reaction and the choline gave a distinct spot with iodine vapor[16] and with phosphomolybdate[13]. The choline also gave a radioactive Reineckate precipitate, which was recrystallized from acetone–propanol without loss of specific activity.

The identities of mono- and dimethylaminoethanol were further verified by the following tests: The radioactive compounds were eluted from the paper chromatogram with water. Approx. 100 μmoles of the appropriate compounds were added along with 1 ml of 1 *N* sodium hydroxide and 0.8 ml of 1 *M* methyl iodide in ethanol. The mixtures were shaken for 1 h at 45°. The choline formed was precipitated as the Reinecke salt and recrystallized three times from acetone–propanol. The specific activity of the choline Reineckate formed from monomethylaminoethanol was 11, 9 and 10 counts/min/mg after one, two and three recrystallizations respectively and from dimethylaminoethanol 67, 66 and 68 counts/min/mg. In another experiment carrier choline and Reinecke salt were added to samples of isolated radioactive mono-

and dimethylaminoethanol. In this instance, completely inactive choline Reineckates were obtained after one recrystallization from acetone–propanol.

Phosphoaminoethanol and phosphocholine

These materials were chromatographed in two different paper systems: Propanol–80% formic acid–water (6:3:1) (R_F values 0.15 and 0.30) and water saturated phenol–80% formic acid (99:1) (R_F values 0.40 and 0.85). The isolated, radioactive compounds moved identically with the synthetic reference compounds in the two systems. Both compounds gave positive spot tests for phosphate[17]. The phosphoaminoethanol gave a positive ninhydrin reaction, the phosphocholine a distinct spot with iodine vapor[16].

Cytidinediphosphoaminoethanol and -choline

These compounds were identified by their u.v. absorption spectra and by paper chromatography. The fractions containing cytidinediphosphoaminoethanol gave an absorption spectrum identical with that of cytidine both in acid and alkaline solution. When chromatographed on paper with 0.25 *M* TRIS–HCl buffer, pH 8–ethanol (1:1), and with 0.02 *M* acetic acid–ethanol (4:6), only one u.v. absorbing spot was found with R_F values of 0.71 and 0.50 in the two systems respectively compared to 0.69 and 0.33 reported by KENNEDY AND WEISS[6]. In animals injected with [^{14}C]aminoethanol, the compound became strongly radioactive.

The fractions containing cytidinediphosphocholine gave a somewhat distorted absorption spectrum compared to cytidine, due to some contamination from a neighboring peak in the column chromatogram. When chromatographed on paper with TRIS buffer–ethanol and with acetic acid–ethanol, one major spot was found with R_F values of 0.64 and 0.55 in the two systems respectively, compared to the values 0.59 and 0.36 reported by KENNEDY AND WEISS[6]. When eluted from the paper, the major spot gave the typical u.v. absorption spectrum of cytidine.

The cytidinediphosphocholine was further identified by acid hydrolysis and paper chromatography of the degradation products. The compound was refluxed with 1 *N* HCl for 1 h. By subsequent chromatography on paper with propanol–formic acid–water, the only radioactive spot found had the same R_F value as phosphocholine. It also gave a positive spot reaction for phosphate.

Degradation of choline

To determine the distribution of ^{14}C between the methyl- and the hydroxyethyl groups of choline, the compound was degraded by alkaline permanganate oxidation as described by DU VIGNEAUD *et al.*[3]. Samples of the choline and of the trimethylamine formed during the oxidation were precipitated as chloroplatinates. From the specific activities the distribution of ^{14}C between choline methyl and hydroxyethyl groups was calculated.

Determination of specific activities

The specific activity of choline was determined as explained above. The specific activity of cytidine diphosphocholine was calculated from the radioactivity and the u.v. absorption of the peaks in the column chromatograms. The calculated values must be considered as minimum values, due to the slight contamination of u.v.-absorbing material from a neighboring peak in the chromatograms.

In the experiments with [^{14}C]serine, the specific activity of cytidinediphosphoaminoethanol could not be calculated directly from the column chromatograms. The large amount of radioactivity ($\sim$ 100 μC) injected into the animals led to a relatively high "background" radioactivity in all the column fractions, and no distinct radioactive peak corresponding to cytidinediphosphoaminoethanol was detectable. A rough estimation of the specific activity was obtained by chromatographing the isolated cytidinediphosphoaminoethanol on paper with acetic acid–ethanol (see above), eluting the u.v.-absorbing spot from the paper and measuring the absorption and the radioactivity in the eluate. Only 4–6 counts/min on the planchets were obtained. The calculated values therefore must be considered as only rough approximations.

The specific activities of aminoethanol, phosphoaminoethanol and of serine were calculated on the basis of quantitative determinations of these compounds with ninhydrin according to MOORE AND STEIN[18].

RESULTS

Table I shows the results of an experiment in which the total radioactivity incorporated into phosphoaminoethanol, phosphocholine, phospholipid aminoethanol and phospholipid choline was measured after the injection of [1,2-^{14}C]aminoethanol, [1,2-^{14}C]phosphoaminoethanol and [^{14}CH$_3$]choline. It is seen that injection of both choline and aminoethanol (and phosphoaminoethanol) led to the appearance of radioactive choline in the phospholipids, whereas only choline gave measurable activity in the water soluble phosphocholine.

These results indicated that phosphocholine is not an intermediate in the biosynthesis of lecithin from aminoethanol, and we therefore had to consider cytidinediphosphoaminoethanol or phosphatidylaminoethanol as the possible methyl acceptors.

In the experiments shown in Figs. 3 and 4, [^{14}CH$_3$]methionine was chosen as the labeled precursor, because the cytidinediphosphoaminoethanol and -choline peaks appear very close together in the column chromatogram. As a consequence it was

TABLE I

TOTAL RADIOACTIVITIES OF COMPOUNDS ISOLATED FROM RAT LIVER AFTER THE INTRAPERITONEAL INJECTION OF LABELED AMINOETHANOL, PHOSPHOAMINOETHANOL, OR CHOLINE

The animals were sacrificed 2 h after the injections.

Compound injected	*Radioactivity of compounds isolated from liver in counts/min*			
	Phosphoaminoethanol	*Phosphocholine*	*Phospholipid aminoethanol*	*Phospholipid choline*
Approximately 10 μmoles [1,2-^{14}C]aminoethanol, 2.5·10^6 counts/min	13,500	Not detectable	280,000	65,000
Approximately 10 μmoles [1,2-^{14}C]phosphoaminoethanol, 3·10^6 counts/min	25,000	Not detectable	370,000	58,000
Approximately 10 μmoles [^{14}CH$_3$]choline, 2.5·10^6 counts/min	—	130,000	—	215,000

difficult to secure reliable measurements of the cytidinediphosphocholine radioactivity in the vicinity of a highly radioactive cytidinediphosphoaminoethanol peak.

The data from chromatograms of the phospholipid bases, isolated after the injection of radiomethionine, favored phosphatidylaminoethanol as the methyl acceptor. Fig. 1 shows that radioactive mono- and dimethylaminoethanol, the presumed intermediates in choline biosynthesis, were found in the phospholipids. Fig. 3 further shows that these compounds have a high turnover rate compared to what is known of other phospholipid bases. The methylation of phosphatidylaminoethanol is further supported by the finding that the specific radioactivity of cytidinediphosphocholine seemed to reach its maximum later than did the phospholipid choline (Fig. 4). Admittedly, the accuracy of the determination of cytidinediphosphocholine's specific activity was poor. This was calculated from counting rates of 20–100 counts/min on the planchets. On the other hand, considering the relative pool sizes of cytidinediphosphocholine and of phosphatidylcholine in rat liver, the former evidently would have a specific activity many times that of phospholipid choline, if it were an intermediate.

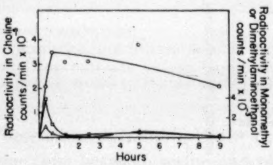

Fig. 3. Time curves for the appearance of radioactivity in phospholipid monomethylaminoethanol, dimethylaminoethanol and choline after the intraperitoneal injection of approx. 6 μmoles [$^{14}CH_3$]methionine, $1.9 \cdot 10^6$ counts/min. △, monomethylaminoethanol; □, dimethylaminoethanol; ○, choline.

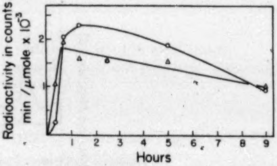

Fig. 4. Time curves for specific radioactivity of cytidinediphosphocholine and phospholipid choline after the intraperitoneal injection of approx. 6 μmoles [$^{14}CH_3$]methionine, $1.9 \cdot 10^6$ counts/min. ○, cytidinediphosphocholine; △, choline.

Our conclusions from these *in vivo* experiments have been verified by *in vitro* experiments. Fig. 5 shows that incubating isolated microsomes with [$^{14}CH_3$]adenosylmethionine led to the formation of radioactive phospholipidcholine (Fig. 5B), and that this choline was bound to lecithin (Fig. 5A).

In another *in vitro* experiment, the extracted radioactive phospholipids were treated with *Clostridium welchii* toxin in ether–alcoholic solution according to the procedure of HANAHAN AND VERCAMER[19]. From the reaction mixture, radioactive phosphocholine was isolated by paper chromatography, in the two systems used for this compound (see above) confirming the identity of the radioactive lipid as lecithin.

The "cephalin" peak in the phospholipid chromatogram from this *in vitro* experiment also contained some radioactivity. This activity was shown to be due to dimethylaminoethanol after hydrolyzing a sample of the isolated "cephalin" with hydrochloric acid and chromatographing the hydrolyzate on paper in the phenol-butanol–formic acid system (see above). The small amount of radioactive monomethylaminoethanol present in the phospholipid hydrolyzate, could not be localized in the phospholipid chromatogram.

Fig. 6 shows the labeling of liver phospholipid bases after the injection of [3-^{14}C]-

serine. Only a very small fraction ($< 0.5\%$) of the injected radioactivity was incorporated, compared to the degree of incorporation of radioactive aminoethanol, choline or methionine-methyl (Table I, Fig. 3). The turnover time of phosphatidylserine in rat liver is known to be of the same order as that of the other phosphatides[20]. This low incorporation is probably due to extensive dilution of the injected serine by internal, inactive serine.

The striking feature in this experiment is that the phospholipid serine reached its maximum activity within 40 min, whereas the phospholipid aminoethanol activity kept on increasing for at least 5 h after the serine injection. The same gradual increase was found for the choline hydroxyethyl group. The same feature is evident in the methionine experiment, where the aminoethanol activity rose linearly through all the 9 h of the experiment (Fig. 7).

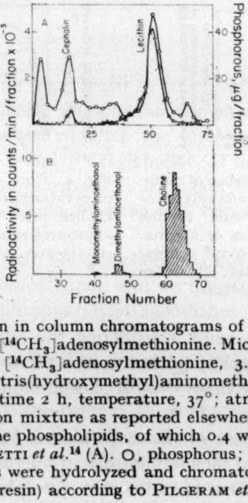

Fig. 5. Radioactivity distribution in column chromatograms of phospholipids isolated from rat-liver microsomes incubated with [$^{14}CH_3$]adenosylmethionine. Microsomes from approximately one rat liver were incubated with [$^{14}CH_3$]adenosylmethionine, 3.5 µmoles = 1·10^6 counts/min; reduced glutathione, 17 µmoles; tris(hydroxymethyl)aminomethane buffer, pH 7.25, 250 µmoles. Total volume: 6 ml, incubation time 2 h, temperature, 37°; atmosphere, air. The phospholipids were isolated from the incubation mixture as reported elsewhere[11]. Approx. 10% of the added radioactivity was recovered in the phospholipids, of which 0.4 were chromatographed on a silicic acid column according to MARINETTI *et al.*[14] (A). ○, phosphorus; △, radioactivity. Approximately 0.6 of the isolated phospholipids were hydrolyzed and chromatographed on a Dowex 50 (strong anionic resin) according to PILGERAM *et al.*[2] (B).

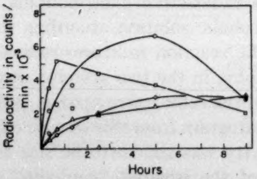

Fig. 6. Time curves for the appearance of radioactivity in phospholipid serine, aminoethanol and choline hydroxyethyl- and choline methyl groups after the intraperitoneal injection of 6 µmoles [3-^{14}C]serine, 3.8·10^6 counts/min. □, serine; ◇, aminoethanol; △, choline-hydroxyethyl; ○, choline-methyl.

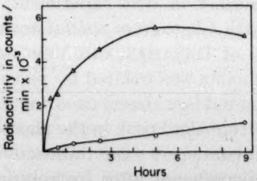

Fig. 7. Time curves for the appearance of radioactivity in liver phospholipid serine and -aminoethanol after the intraperitoneal injection of approximately 6 µmoles [$^{14}CH_3$]methionine, 1.9·10^6 counts/min. △, serine; ○, aminoethanol.

TABLE II

TOTAL RADIOACTIVITIES AND SPECIFIC RADIOACTIVITIES OF COMPOUNDS ISOLATED FROM LIVER OF RATS INJECTED INTRAPERITONEALLY WITH APPROXIMATELY 49 μmoles [3 ^{14}C]SERINE*

	Total radioactivity in counts/min in liver phospholipid bases			*Total counts/min in aqueous liver extract*	*Specific radioactivity of phospholipid bases (counts/min/µmole)*		*Specific radioactivity of compounds in aqueous liver extract (counts/min/µmole)*	
	Serine	*Amino-ethanol*	*Choline***	*Phospho-amino-ethanol*	*Serine*	*Amino-ethanol*	*Phospho-amino-ethanol*	*Cytidine-diphospho-amino-ethanol****
Animal sacrificed after 2 h	35,700	8,500	39,800	1,850	3,650	140	—	~ 340
Animal sacrificed after 4 h	34,700	16,600	57,450	2,500	3,750	290	320	~ 380

* $31 \cdot 10^6$ counts/min.

** Determination of intramolecular distribution of radioactivity not performed.

*** Only approximate values based on counting rates of 4–6 counts/min. Not corrected for possible radioactivity in the cytidine portion of the molecule.

These observations indicate that there must be an intermediate with a long turnover time between serine and phosphatidylaminoethanol.

To elucidate further the mechanism of this conversion, another experiment was undertaken, in which a larger amount of [3-^{14}C]serine was injected to overcome the internal dilution (Table II). In this experiment the aqueous extracts of the livers were chromatographed to isolate the phosphoaminoethanol and the cytidinediphosphoaminoethanol. A significant radioactive peak was found corresponding to cytidinediphosphocholine, but no distinct peak corresponding to cytidinediphosphoaminoethanol. There was also a small peak corresponding to phosphoaminoethanol. As mentioned in METHODS, the cytidinediphosphoaminoethanol isolated by paper chromatography was also found to be slightly radioactive. The small amount of the compound present permitted only a rough calculation of its specific activity. The specific activity of phosphoaminoethanol could be calculated more accurately, because a larger amount of this compound was present.

As seen from Table II, the water soluble derivatives of aminoethanol were found to have a somewhat higher specific activity than the phospholipid aminoethanol. At first sight, therefore, this experiment does not seem to exclude these compounds as being intermediates in the formation of phosphatidylaminoethanol from serine. We consider it unlikely that these compounds are intermediates for the following reasons: From our experiments it is evident that the specific activity of phosphatidylaminoethanol keeps on rising for at least 5 h after the injection of radioserine. During this time the immediate precursor must have a higher specific activity. Considering then the sizes of the metabolic pools in rat liver of phosphoaminoethanol (approx. 5 μmoles), cytidinediphosphoaminoethanol (< 1 μmoles) and of phosphatidylaminoethanol (> 50 μmoles), and the turnover time of many hours of the latter compound, one would expect the immediate precursor to have a specific activity many times greater than that of the phosphatidylaminoethanol. This is evidently not so. A more reasonable explanation, therefore, of our findings, is that the turnover of the phosphatidylaminoethanol is not uniform, but that the breakdown of a more active phospholipid fraction gives rise to the phosphoaminoethanol and cytidine-diphosphoaminoethanol with a specific activity somewhat higher than the average aminoethanol specific activity.

DISCUSSION

The results of the *in vivo* experiments indicate that the decarboxylation of serine and the methylation of aminoethanol take place after these compounds have been incorporated into the phospholipids. The methylation of phosphatidylaminoethanol is further supported by *in vitro* experiments. We have also shown that S-adenosylmethionine is the donor of all the methyl groups in choline[11]. On this basis therefore, we suggest the metabolic cycle shown in Fig. 8, showing the interrelationship between phosphatide- and methyl group metabolism. The reactions on the right side of the diagram, involving compounds freely soluble in water, are well known, and all of them have been shown to take place *in vitro*[21]. This is also true of the synthesis of methionine from formaldehyde or serine[22–24], although the exact mechanism in this conversion is not known. Numerous *in vivo* experiments[25,26], and recently also *in vitro* experiments with enzymes from microorganisms[27,28], strongly suggest that vitamin B_{12} is a cofactor in the *de novo* synthesis of methionine methyl groups. There is also evidence for an oxidative demethylation of methionine[29].

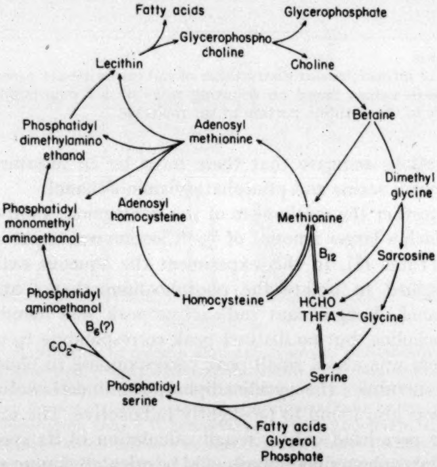
Fatty acids
Glycerophosphate
Glycerophospho choline
Lecithin
Choline
Betaine
Phosphatidyl dimethylamino ethanol
Adenosyl methionine
Dimethyl glycine
Phosphatidyl monomethyl aminoethanol
Adenosyl homocysteine
Methionine
B_{12}
Sarcosine
Phosphatidyl aminoethanol
Homocysteine
HCHO
THFA
Glycine
B_6(?)
CO_2
Serine
Phosphatidyl serine
Fatty acids
Glycerol
Phosphate

Fig. 8. Proposed metabolic pathways for metabolism of phospholipids and methyl groups.

The exact mechanism for serine incorporation into phosphatides is not known, but a recent report by Dils and Hübscher[7] indicates a mechanism different from that reported by Kennedy and Weiss for aminoethanol and choline[6]. Dils and Hübscher found "subcellular" particles to incorporate serine into phospholipids in the presence of calcium ions and phosphate. They also found that choline is incorporated under the same conditions, a result which indicates the existence of a third pathway for lecithin biosynthesis in addition to the pathway reported by Kennedy and Weiss, and the one reported in this paper.

It has been shown that serine is converted to aminoethanol[1], and also that aminoethanol[2], mono- and dimethylaminoethanol[30] can serve as precursors for choline. It is, however, a striking fact that, except for recent work by STEKOL *et al.*[4], the reaction products have always been isolated from the phospholipids, a fact which to a certain extent supports our conclusion that these reactions represent interconversion of phospholipids. The accumulation of mono- and dimethylaminoethanol in the phospholipids of choline-requiring mutant strains of *Neurospora crassa*[31], also fits in with the proposed metabolic scheme.

STEKOL *et al.*[4] have reported the isolation of free choline after incubating dimethylaminoethanol and adenosylmethionine with rat-liver homogenate. At present, it is not possible to explain with certainty the differences between their and our results. One possibility is that the free choline isolated in their experiments, represents a hydrolytic product of lecithin, which therefore might be the primary product. The low yield and the use of relatively large amounts of liver homogenates may indicate such an explanation. Their report also contains no experimental data showing the requirement for free dimethylaminoethanol in the formation of the choline isolated. In *in vivo* experiments with folic acid-deficient rats[5], these workers obtained results indicating that the first two methyl groups of choline are formed by a folic acid-dependent mechanism and that the third methyl group is transferred from adenosylmethionine. Our *in vitro* experiments do not support this theory.

In turnover studies it has been found that glycerophosphoaminoethanol and glycerophosphocholine behave as breakdown products from the phospholipids[12], and further that glycerophosphocholine is broken down to glycerophosphate and choline[32]. FAIRBAIRN[33] reported that free fatty acids are probably the primary breakdown products of lecithin. Thus there is good experimental support for all the steps in the proposed metabolic cycle.

The cycle represents a new pathway for lecithin biosynthesis. Experiments by GROTH *et al.*[34] suggest that this pathway is quantitatively more important in the rat than the pathway(s) involving utilization of free choline. These workers compared the incorporation of [$^{14}CH_3$]choline and [1,2-^{14}C]choline into phospholipids in rats. They found that the methyl-labeled choline is twice as efficient as the hydroxyethyl-labeled choline as a lecithin precursor both in liver and in brain. Considering the fact that only one of the three methyl groups of choline is reutilized by direct transmethylation by way of betaine to methionine, while the two other methyl groups are oxidized and probably are subject to a high dilution in the "formaldehyde–formate pool", and are therefore poorly reutilized for methyl synthesis (*cf.* the low conversion to choline methyl of the serine-3-carbon compared to that of methionine methyl in our experiments), it can be said almost with certainty that this new pathway is very important in the normal rat.

GROTH *et al.*[34] also found different half-lives for the lecithins formed from the two choline precursors, indicating different turnover rates for the lecithins formed by the two pathways. In our experiments, the specific activities of cytidinediphosphoaminoethanol and -choline were found to rise above the average specific activities of phospholipid aminoethanol and -choline. This may also indicate a non uniform turnover of the phosphatides.

Further, the two pathways seem to have different intracellular localizations. The cytidine-dependent incorporation of choline into phospholipid is reported to take place in the mitochondria[6]. We have found that the methylation of phosphatidylaminoethanol takes place in the microsome fraction[11]. In this connection it is worth noticing that the acylation of glycerophosphate to phosphatidic acid[35] and the synthesis of neutral fat[36] also occur in the microsome fraction of the cell. Consequently the methylation of the phosphatidylaminoethanol in the microsomes may prove to be a finding of considerable importance, and the possibility should be given consideration that the proposed "phospholipid cycle" represents a mechanism by which neutral fat and long chain fatty acids are transported into the mitochondria for utilization (see also ARTOM[37]).

ACKNOWLEDGEMENTS

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THE PARTIAL PURIFICATION AND PROPERTIES OF INDOLE-3-GLYCEROL PHOSPHATE SYNTHETASE FROM *ESCHERICHIA COLI*

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SUMMARY

The enzyme which converts 1-*o*-carboxyphenyl-1-deoxyribulose-5-phosphate (anthranilic deoxyribulotide) to indole-3-glycerol phosphate has been partially purified from cell extracts of *Escherichia coli* and some of its properties examined. By growing tryptophan auxotrophs on media in which the available tryptophan was limited, crude extracts were obtained with up to 60 times the specific activity of similar extracts from wild type *E. coli*. A further 17-fold increase in specific activity was achieved by acetone precipitation followed by ammonium sulphate fractionation.

Experiments employing the mixing of fractions obtained during purification and the mixing of crude extracts of a number of tryptophan auxotrophs of *E. coli* strongly suggest that the conversion of anthranilic deoxyribulotide to indole-3-glycerol phosphate is carried out by a single enzyme.

A number of compounds with structural similarities to the substrate were tested as possible inhibitors of indole-3-glycerol phosphate synthetase. Anthranilic acid and certain of its derivatives were found to inhibit the enzyme.

INTRODUCTION

A number of tryptophan auxotrophs of *Escherichia coli*, *Aerobacter aerogenes* and *Salmonella typhimurium* have been found to accumulate 1-*o*-carboxyphenylamino-1-deoxyribulose[1,2]. Cell-free extracts of such strains convert anthranilic acid to 1-*o*-carboxyphenylamino-1-deoxyribulose-5-phosphate (anthranilic deoxyribulotide) but not to indoleglycerol phosphate, while extracts from other tryptophan auxotrophs contain an enzyme (or enzymes) which convert anthranilic deoxyribulotide to InGP[2].

Abbreviations: InGP, indole-3-glycerol phosphate; DEAE, diethyl-aminoethyl; Tris, tris-(hydroxymethyl)aminomethane.

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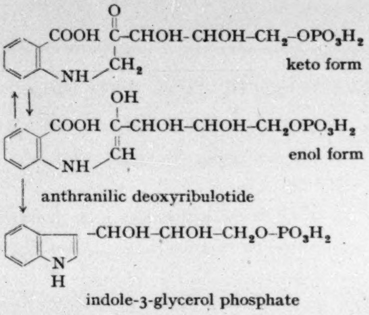

COOH C–CHOH–CHOH–CH₂–OPO₃H₂
CH₂
NH
keto form
OH
COOH C–CHOH–CHOH–CH₂OPO₃H₂
CH
NH
enol form
anthranilic deoxyribulotide
–CHOH–CHOH–CH₂O–PO₃H₂
N
H
indole-3-glycerol phosphate

The object of the present communication is to report on the partial purification of this enzyme, for which the name indole-3-glycerol phosphate synthetase (InGP synthetase) is proposed, and on some of its properties. As the overall reaction involves the removal of both CO_2 and H_2O and ring closure, it was of particular interest to examine whether one or more enzymes were involved in the conversion.

MATERIALS AND METHODS

Organisms

A number of tryptophan auxotrophs of *E. coli* were employed in this study. In most experiments *E. coli* T-8, described by YANOFSKY[3] was used. This strain and the 14 strains of *E. coli* employed in the extract-mixing experiments were all derived from *E. coli* K-12. The wild type *E. coli* 518 and the tryptophan auxotroph (7-4) derived from it have been described by GIBSON, JONES AND TELTSCHER[4]. All the auxotrophs used were obtained by penicillin selection following u.v. irradiation.

Chemicals

All the chemicals used with the exception of anthranilic deoxyribulotide were obtained commercially and were not purified further. Chromatography on DEAE cellulose was carried out using "Selectacel", DEAE Reagent Grade, obtained from Brown Company, Berlin, New Hampshire, U.S.A.

The synthesis of anthranilic deoxyribulotide has been described[2]. Briefly the sodium salt of ribose-5-phosphate was heated with an excess of anthranilic acid in the presence of ethyl malonate. Excess anthranilic acid was then removed by three successive extractions with ethyl acetate and the solvent in the aqueous layer removed by gentle warming under vacuum. The resulting aqueous solution was kept at —15° and used as the stock solution of anthranilic deoxyribulotide. The yield of anthranilic deoxyribulotide, as assayed by the amount of InGP formed by InGP synthetase was about 10 % of theoretical based on the amount of ribose-5-phosphate in the reaction mixture. Although a large excess of ribose-5-phosphate was presumably present in the solution of anthranilic deoxyribulotide it appeared to have no inhibitory effects on the reaction studied.

Buffers: These were Tris–HCl mixtures and mixtures of potassium phosphates.

Estimation of InGP synthetase activity

The incubation mixture for studying the conversion of anthranilic deoxyribulotide to InGP contained the following in a final volume of 0.5 ml. Anthranilic deoxyribulotide (0.22 μmoles), Tris buffer (pH 8.8, 25 μmoles) and enzyme solution made up to the final volume with distilled water and any other additions. Tests were incubated for 20 min at 37°.

The reaction was stopped by the addition of 0.2 ml of *M* acetate buffer (pH 5). This was followed immediately by 0.5 ml of 0.2 *M* aqueous sodium meta periodate to quantitatively convert the InGP formed to indole-3-aldehyde[5]. The mixture was allowed to stand at room temperature for 20 min after which 0.4 ml of *N* NaOH and 5 ml of ethyl acetate were added. After shaking 10–20 times to extract the indole-3-aldehyde the tubes were centrifuged at low speed for a few minutes and the O.D. at 290 mμ of the ethyl acetate layers measured against an ethyl acetate blank. The extinction coefficient of indole-3-aldehyde at 290 mμ is 11,400.

The unit of enzyme activity

One unit of enzyme activity is taken as that amount of enzyme required to form 0.1 μmole of InGP in 20 min at 37°. The use of this unit enables direct comparison with the next sequentially related enzyme, tryptophan synthetase. Specific activities are expressed as the units of enzyme activity /mg of protein. Protein was determined with FOLIN's phenol reagent[6].

Preparation of cell extracts

The cells were grown on the citric acid–mineral salts mixture described by VOGEL AND BONNER[7] supplemented with 0.16% glucose and 0.005% acid hydrolysed casein (neutralized before use). Unless otherwise stated 2 μg/ml of indole was included in the medium for the tryptophan auxotrophs.

For small batches, 1-l quantities of medium were inoculated with a 6–8 h culture in nutrient broth to give an initial cell population of about 10^6/ml. The cultures were then vigorously shaken for 16 h at 37°. For a large batch, 15 flasks each containing 1 l were prepared as above and used as inoculum for 45 l of fresh warm medium in a stainless steel tank and vigorously aerated at 37° for 7.5 h.

The grown cultures were harvested using a Sharples super centrifuge and the cells washed once with cold 0.9% NaCl. After resuspending in 0.1 *M* Tris buffer, pH 7.8 to give a final cell concentration of about $4 \cdot 10^{11}$ cells/ml the cells were disrupted for 20 min in a 10 kc Raytheon sonic oscillator. Cell debris was removed by centrifuging at 144,000 × *g* in a Spinco Model L centrifuge for 30 min. The supernatant fluid (crude extract) was stored at —15°. Such extracts had a protein content of about 20 mg/ml.

Enzyme preparations

Unless otherwise stated the enzyme preparation used was obtained by collecting the protein precipitated from a crude extract by ammonium sulphate fractionation (26 to 40%) The conditions under which such a precipitation was carried out are described in the section on purification.

RESULTS

Purification by fractionation

The most important factor in obtaining InGP synthetase preparations with a high specific activity was limiting the amount of tryptophan available for growth of tryptophan auxotrophs of *E. coli*. As can be seen from Table VII under these conditions crude extracts are obtained with a specific activity about 60 times as high as that of a corresponding extract made from wild type *E. coli*.

Attempts were made to purify the InGP synthetase from *E. coli* T-8 extracts by first removing nucleic acid and then using ammonium sulphate precipitation acetone precipitation, chromatography on DEAE cellulose, acid precipitation, heat, treatment and adsorption on calcium phosphate gel. The last 3 procedures have not as yet proven very useful and will not be described.

The most successful method of purification found so far is a combination of acetone and ammonium sulphate precipitations. As can be seen from the experiments summarized in Table I, it was most important to use the acetone precipitation as the first step. Attempts to follow ammonium sulphate fractionation with acetone resulted in poor recoveries and no significant purification.

TABLE I

FRACTIONATION OF STREPTOMYCIN-TREATED EXTRACT WITH ACETONE AND AMMONIUM SULPHATE

The fractions given are the best of a series taken in each case. For details of ammonium sulphate and acetone fractionations see text. Recoveries based on streptomycin-treated extract.

	Fraction	
	$(NH_4)_2SO_4$ 27–45%	*Acetone 25–50%*
Specific activity	11	14.5
% recovery of units of activity	50	90
	Further treatment of fraction above with	
	Acetone 70–80%	*$(NH_4)_2SO_4$ 27–45%*
Specific activity	17	70
% recovery of units of activity	5	56

By taking only the protein precipitated by higher concentrations of acetone it was possible to obtain enzymes of higher purity but only at the expense of overall yield. In Table II the purification procedure which gave the highest specific activity of InGP synthetase is described. This purification represents a 17-fold increase in specific activity over the starting material, the crude *E. coli* T-8 extract, and 1000-fold over that in extracts of the wild type *E. coli* K-12.

The details of the experiment summarized in Table II are as follows. All operations were carried out at 0–5°. To remove nucleic acids, one-half volume of 20% streptomycin sulphate was added with stirring to *E. coli* T-8 crude extract. Stirring was continued for 10 min after which the mixture was centrifuged at 4,600 × *g* for 10 min. The supernatant was decanted and stored at —15°. 10 ml of treated

TABLE II

PURIFICATION OF INDOLEGLYCEROL PHOSPHATE SYNTHETASE FROM *E. coli* T-8

For details of experiment see text.

	Specific activity	*Purification*	*% units recovered*
Crude extract	10	—	100
Streptomycin-treated and dialyzed*			104
Acetone fractionation			
(a) 0–40 %	5	0.5	39
(b) 40–50 %	17	1.7	26
Ammonium sulphate fractionation of (b)			
30–45 %	84	8.4	5
45–55 %	170	17	6.7

* The protein content after streptomycin treatment could not be determined accurately because residual streptomycin interfered with the protein assay.

extract was dialysed against 1 l of 0.03 *M* Tris buffer pH 7.8 for 2 h changing the buffer after 1 h. The final volume was 11.7 ml and to 10 ml stirred in an ice bath was added 4 ml of acetone, chilled to —15°, over a period of about 0.5–1 min. After further stirring for 1 min the precipitate was collected by centrifugation at 12,000 × *g* for 2 min. The precipitate was taken up in 10 ml of 0.1 *M* Tris buffer pH 7.8 (0–40 % acetone fraction). To the supernatant was added a further 1-ml portion of cold acetone as above and the precipitated protein centrifuged and taken up in 5 ml Tris buffer (40–50 % acetone fraction).

To 4 ml of the 40–50 % acetone fraction 0.87 g $(NH_4)_2SO_4$ (30 % saturation) was added, the resulting solution stirred for 10 min and then centrifuged at 4,600 × *g* for 10 min. The precipitate was taken up in 4 ml 0.1 *M* Tris pH 7–8 and $(NH_4)_2SO_4$ added to the supernatant to give 45 % saturation. After collecting the 30–45 % fraction a further fraction at 45–55 % saturation was collected.

Chromatography

Although it has not yet been possible to obtain appreciable purifications of InGP synthetase by chromatography on DEAE cellulose columns, nevertheless this procedure was employed to obtain fractions for the mixing experiments to be described later. A crude extract of *E. coli* 7-4 (25 ml) was prepared for the column by treatment with $MnCl_2$ (1.5 ml *M* solution/20 ml extract) to remove nucleic acids and then precipitating the enzymes with 50 % saturated $(NH_4)_2SO_4$. The precipitated enzymes were dissolved in 0.1 *M* Tris buffer pH 7.8 and dialysed for 2 h against 0.01 *M* phosphate buffer pH 7.8 plus 10^{-4} glutathione and $2 \cdot 10^{-5}$ *M* pyridoxal phosphate. The latter compounds were added to protect the component B of tryptophan synthetase[8] which was being collected at the same time and they were also included in the eluting buffers. A column of DEAE cellulose 2 cm × 90 cm was used and the linear gradient elution system was 1 l of 0.01 *M* phosphate buffer pH 7.0 in the mixing bottle and an equal volume of 0.4 *M* phosphate pH 8.0 in the inlet bottle. Fractions of 7.5 ml were collected every 18 min and the buffer changed at fraction 173 to elute the B component of tryptophan synthetase. Fig 1 shows that active material was eluted as a well-defined peak.

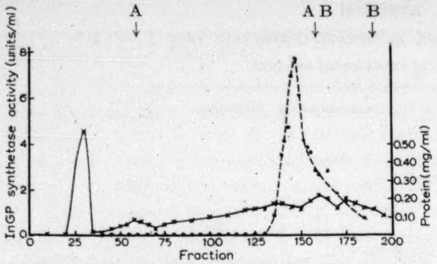


Fig. 1. Chromatography of InGP synthetase on DEAE column. Extract from *E. coli* 7-4. Positions of A and B proteins of tryptophan synthetase as indicated. For details of experiment see text. ●—●, InGP synthetase activity (units/ml); ×—×, protein (mg/ml).

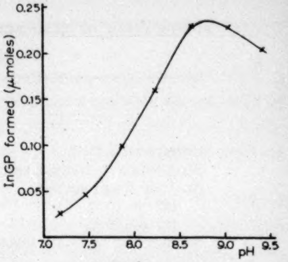


Fig. 2. Effect of pH on activity of InGP synthetase in Tris buffer. For details of test systems (anthranilic deoxyribulotide, 0–6 μmole; InGP synthetase and buffer) see METHODS.

Inhibitors

It had been shown previously[9] that when cultures of *E. coli* are grown in the presence of subinhibitory concentrations of 3-methyl anthranilic acid, anthranilic deoxyribulotide accumulates in the culture medium. This finding indicated that 3-methyl anthranilic acid probably inhibited indoleglycerol phosphate synthetase and using the enzyme assay system described above it was possible to test this directly.

The results of the tests with this compound and with other derivatives of anthranilic acid are shown in Table III. It can be seen that the anthranilic acid analogues fell into 2 groups when tested at a concentration of 10^{-3} *M*, those showing appreciable inhibition and those with little or no effect.

Other inhibitors which were tested in the system given in Table III and the concentrations used together with percentage inhibition are as follows: *p*-chloromercuribenzoate (10^{-5} *M*; 77 %) Versene ($5 \cdot 10^{-2}$ *M*; 32 %; 10^{-2} *M*, 6 %), hydroxylamine (10^{-3} *M*, 17 %; 10^{-4} *M*, 6 %).

Effect of pH on IGP synthetase activity

In Tris buffer a well-defined optimum was shown at pH 8.8 as illustrated in Fig. 2. In phosphate buffer, tested over the range pH 6.5–8.0, the activity shows a steady increase and while at pH 8.8 the activity is greater in Tris than in phosphate buffer, between pH 7 and 8 the activity is much higher in phosphate buffer than in Tris.

Effect of temperature on activity

The enzyme shows a Q_{10} of about 2 between 20° and 30° and there is further increase in activity to 37°. When heated in 0.1 *M* Tris buffer pH 7.8 at 45° for 10 min 75 % of the activity is lost.

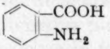
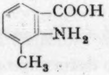
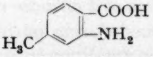
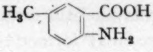
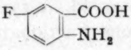
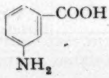
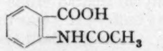
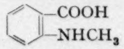
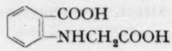
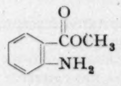
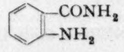
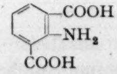
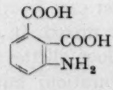
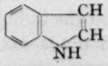
Stability of enzyme

Solutions of IGP synthetase stored at —15° seem to be quite stable. The ammonium sulphate precipitated preparation used for most of the experiments described

TABLE III

INHIBITION OF INDOLEGLYCEROL PHOSPHATE SYNTHETASE BY ANTHRANILIC ACID AND RELATED COMPOUNDS

E. coli T-8 preparation (see METHODS) was incubated in the assay mixture (see text) with and without the inhibitors shown.

Compound	*Structure*	*% inhibition by 10^{-3} M*
Anthranilic acid		31
3-methyl anthranilic acid	 COOH NH_2 CH_3	40
4-methyl anthranilic acid	 COOH NH_2 H_3C	36
5-methyl anthranilic acid	 H_3C COOH NH_2	25
5-fluoro anthranilic acid		27
3-amino benzoic acid		3
N-acetyl anthranilic acid	 COOH $NHCOCH_3$	2
N-methyl anthranilic acid		5
Phenylglycine *o* carboxylic acid	 COOH $NHCH_2COOH$	10 (stimulation)
Methyl anthranilate		0
2-amino benzamide		0
2-amino isophthalic acid	 COOH NH_2 COOH	4
3-amino phthalic acid	 COOH COOH NH_2	7 (stimulation)
Indole		5

here showed no decrease in activity over a period of more than 3 months, despite repeated freezing and thawing.

Kinetic studies

Examination of the rate of formation of InGP from anthranilic deoxyribulotide showed that the conversion had the characteristics of a first order reaction. Determination of initial velocities of the reaction were made measuring IGP formation at 2, 4 and 6 min at concentrations of anthranilic deoxyribulotide of $8 \cdot 10^{-5}$, $4.4 \cdot 10^{-5}$ and $2.2 \cdot 10^{-5}\,M$. By plotting substrate concentration divided by velocity against velocity a Michaelis constant of $1.75 \cdot 10^{-5}\,M$ was obtained.

Mixing of fractions obtained during purification procedures

Attempts were made to determine whether the reaction under investigation was carried out by a single enzyme or 2 or more enzymes. One approach employed was to mix various fractions obtained during purification and to examine for stimulation. If 2 enzymes were involved in InGP formation and partial separation achieved at any step in purification, then mixing of 2 such fractions would result in more InGP formation than the sum of the InGP formed by each preparation alone. On no occasion was such a stimulation observed. A typical result obtained on mixing fractions obtained following an acetone fractionation is shown in Table IV. Mixtures were made from acetone fractionations (before and after ammonium sulphate fractionation) and following both ammonium sulphate fractionation and chromatography on DEAE cellulose. In the latter case fractions from the front and trailing edges of the InGP synthetase peak were mixed. In one experiment when the total recovery of enzymic activity was very low, all the fractions were pooled and treated with ammonium sulphate (50 % saturation) but this did not result in a recovery of more activity than was found in all the individual fractions.

TABLE IV

InGP SYNTHETASE ACTIVITY OF FRACTIONS OBTAINED DURING ACETONE FRACTIONATION

Fractionation of streptomycin-treated crude extract (see text). Precipitates all dissolved in the same volume of 0.1 *M* Tris buffer pH 7.8 as the volume of extract fractionated (10 ml) and tested in standard system (see METHODS).

		(µmoles) InGP formed
(a) Crude extract, streptomycin-treated	0.01 ml	1.18
(b) Acetone precipitated fractions (%) (0.01 ml tested)	0–25	0.01
	25–40	0.60
	40–50	0.45
	50–60	0.01
	60–90	0
Total activity		1.07
(c) Mixture of 0.01 ml of each fraction		1.12

The addition of heated crude extract or yeast extract to material collected from the column failed to increase its activity.

Mixing of crude extracts obtained from a number of strains of E. coli

Another method employed in examining whether InGP synthetase was a single enzyme was to mix the crude extracts of a number of different mutants of *E. coli*, each alone unable to carry out the conversion of anthranilic deoxyribulotide to InGP. If this reaction requires 2 enzymes then some mutants may lack one and other mutants lack the second. Mixing of extracts of members of these 2 groups would result in activity for the overall reaction.

Fourteen different mutants of *E. coli* K-12 were chosen, each judged by nutritional and accumulation tests to be blocked between anthranilic deoxyribulotide and InGP. All of these when tested individually were found to be devoid of InGP synthetase activity. These mutants were grown under the conditions described for the preparation of extracts of *E. coli* T-8 and therefore were likely to contain a high concentration of tryptopahn synthetase. The presence of any serine in the crude extracts would result in the conversion of any InGP formed to tryptophan. The formation of tryptophan was prevented by the inclusion in the tests of $10^{-4}\,M$ hydroxylamine which inhibits this reaction of tryptophan synthetase. An experiment showing the effect of hydroxylamine is given in Table V. A known amount of InGP

TABLE V

THE EFFECT OF HYDROXYLAMINE ON THE REMOVAL OF InGP BY CRUDE EXTRACTS

Basal substrates – Tris buffer pH 8.8, anthranilic deoxyribulotide (for details see METHODS) with additions as shown.

Tube	*1*	*2*	*3*	*4*
E. coli 941 crude extract 0.1 ml	—	+	+	+
InGP synthetase*	+	—	+	+
Hydroxylamine (Final concn. $10^{-4}\,M$)	—	+	—	+
InGP formed (µmoles)	0.10	< 0.005	0.06	0.08

* See METHODS (≡ to about 0.004 ml of crude *E. coli* T-8 extract).

TABLE VI

MIXING OF CRUDE EXTRACTS FROM VARIOUS AUXOTROPHIC STRAINS OF *E. coli* EACH WITH NO InGP SYNTHETASE ACTIVITY

Basal substrates – Tris buffer pH 8.8, anthranilic deoxyribulotide (see METHODS) plus $10^{-4}\,M$ hydroxylamine with additions as shown.

Mixture of extracts of 7 strains of E. coli (0.02 ml each)	*Indole formed (µmoles)*	
	Mixture alone	*Mixture + InGP synthetase**
A	< 0.05	0.10
B	< 0.05	0.10
C	< 0.05	0.10
D	< 0.05	0.10
E. coli T-8 extract (0.004 ml)		0.11

* T-8 extract ((0.004 ml).

synthetase was included in the test. It can be seen that the inclusion of the hydroxylamine did prevent the removal of InGP. The small amount that was removed could be accounted for by the formation of indole from InGP, a reaction which is much slower than the InGP to tryptophan reaction[10] and would be carried out in the presence of hydroxylamine.

The 14 mutant extracts were mixed in groups of 7 at a time so that each extract was tested against every other one. These tests would have detected an activity of 1% of that in *E. coli* T-8 assuming the same specific activity. In no mixture of extracts was there any detectable activity (Table VI). In all experiments a control tube for each mixture was included which contained a known amount of InGP synthetase to detect inhibitory substances in the extracts. No inhibition was detected and the results of one such experiment are summarized in Table VI.

Specific activities of InGP synthetase and related enzymes in crude extracts

It has recently been reported[11] that 4 enzymes concerned in histidine biosynthesis showed co-ordinate repression (release). That is, when mutant or wild-type cells were grown under various conditions the specific activities of the enzymes studied were all increased in the same extract, to a similar extent. The levels of the enzymes of the tryptophan synthetase system, as well as InGP synthetase, were examined in several strains to determine whether co-ordinate repression was also evident in the tryptophan pathway. The specific activities of the enzymes from the various mutants studied are shown in Table VII. It can be seen that the different proteins are not increased equally in the mutants derived from *E. coli* K-12. Thus with *E. coli* A-34 (compared with wild type) IGP synthetase is increased 34-fold, protein A 20-fold and protein B 14-fold. On the other hand, InGP synthetase and protein A in *A. coli* 7-4 seem to be increased to the same extent. From such limited data it appears that co-ordinate repression release may not be an invariable rule.

TABLE VII

SPECIFIC ACTIVITIES OF TRYPTOPHAN SYNTHETASE AND InGP SYNTHETASE OF CRUDE EXTRACTS OF WILD TYPE AND MUTANT STRAINS OF *E. coli*

Specific activity = number of units (0.1 μmole of substrate removed or end product produced in 20 min) per mg protein. Tryptophan synthetase (TS) activity measured in the indole ⟶ tryptophan reaction.

Strain of E. coli	*Specific activity of*		
	InGP synthetase	*Protein A of T.S.**	*Protein B of T.S.**
K_{12} wild type parent	0.167	1.56	1.50
K_{12} (indole)**	0.172	1.41	1.33
T-8	10	***	36
A 34§	5.7	30.8	21.6
518 wild type parent	0.2	1.4	0.85
7-4§	11.7	83	***

* For assay methods see ref. 10, 11.
** Cells grown on medium containing 2 μg indole/ml.
*** Activity not measurable in indole ⟶ tryptophan reaction because of mutation.
§ Cells grown on medium containing 5 μg L-tryptophan/ml.

DISCUSSION

InGP synthetase has proven to be an enzyme which is fairly stable and can readily be assayed in cell-free extracts of *E. coli*. *Escherichia coli* T-8 is a mutant particularly suited to the study of this enzyme since it lacks the A protein of tryptophan synthetase and is therefore unable to convert the product of the reaction, InGP, to either indole or tryptophan. However, with extracts of other types of mutants the interference of the conversion of InGP to tryptophan can be prevented by the inclusion of hydroxylamine which inhibits this reaction, and the small amount of indole formed does not appreciably affect the results.

To obtain enzyme preparations with a high specific activity a most important step is the control of the growth of the tryptophan auxotrophs by limiting the available tryptophan. This method has been employed in the study of other enzymes concerned in tryptophan biosynthesis[2]. In the present case repression release resulted in cell extracts in which there was 60 times as much enzyme per unit protein as that obtained from wild-type cells. Further purification by acetone and ammonium sulphate fractionation gave an additional 17-fold increase in specific activity but only at the expense of a large loss of enzyme activity.

The inhibitory action of anthranilic acid and its analogues on InGP synthetase is of considerable interest as it might have been expected that the analogues would inhibit the metabolism of anthranilic acid but not that of the next intermediate, the deoxyribulotide. This inhibitory effect accounts for the observations of LESTER (see above) on the accumulation of anthranilic deoxyribuloside in the presence of 3-methylanthranilic acid. The other ring-substituted anthranilic acids would not be expected to cause any such accumulation since they are metabolized and probably converted to the corresponding substituted tryptophans. Under the conditions of the cell-free experiments described here, metabolism is not possible and inhibition can be examined. It appears from the inhibition tests with the anthranilic acid analogues that one requirement for inhibition is that both the carboxyl group and the amino group be unsubstituted.

The inhibitory action of anthranilic acid itself on InGP synthetase may explain the ability of this compound to inhibit bacterial growth. RYDON[12] observed that some strains of *Salmonella typhi* were inhibited to some extent by $8 \cdot 10^{-5}\,M$ anthranilic acid. Also it is known that $5 \cdot 10^{-4}\,M$ anthranilic acid will inhibit the growth of a tryptophan auxotroph which will grow well in $10^{-4}\,M$ anthranilic acid (GIBSON, unpublished results).

In the experiments described no requirements for coenzymes or metals have been demonstrated. The addition of yeast extract or heated cell extracts were tried on several occasions but were not found to stimulate InGP formation.

It appears from the findings reported here that the conversion of anthranilic deoxyribulotide to InGP is probably carried out by a single enzyme. The mixing of various fractions obtained during purification did not give any stimulation of activity, indicating that if 2 or more enzymes are involved they fractionate identically. The mixing experiments with extracts obtained from 14 auxotrophic strains of *E. coli* unable to convert anthranilic deoxyribulotide to InGP strongly supports the view that only one enzyme is concerned in the reaction. In a similar application of auxotrophs in the conversion of anthranilic acid to InGP, SMITH AND YANOFSKY[1] were readily able to resolve the overall reaction into a sequence of at least 2 reactions by using mixtures of mutant extracts.

The mechanism of removal of water and carbon dioxide and formation of a pyrrole ring by one enzyme will be an interesting subject for future investigation.

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STUDIES ON THE FUNCTION OF VITAMIN A IN MUCOPOLYSACCHARIDE BIOSYNTHESIS

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SUMMARY

An *in vitro* system of rat colon segments and of rat colon homogenates was elaborated, which incorporated [^{35}S]sulfate and [^{14}C]glucose into mucopolysaccharide.

Mucopolysaccharide was identified by paper chromatography and paper electrophoresis along with chondroitin sulfate carrier; by co-precipitation and dialysis with chondroitin sulfate; by hydrolysis and identification of labeled sulfate or labeled glucosamine.

The homogenate system required adenosine triphosphate, diphosphopyridine nucleotide, glucose and glutamine. The level of incorporation was lowered to about one-half in colon segments and homogenates from vitamin A-deficient rats. Vitamin A, vitamin A-aldehyde and vitamin A-acid (but no other fat-soluble vitamin tested) restored incorporation to normal. With graded levels of vitamin A added, a maximum of incorporation was reached at 10 μg/6 mg of protein in the homogenates. Using glucosamine or galactosamine instead of glucose and glutamine, or uridine diphosphate acetylglucosamine, uridine diphosphate glucuronic acid, uridine diphosphate glucose and acetylglucosamine, incorporation of labeled sulfate was still dependent on vitamin A. This fact shows that the vitamin may be required either in the polymerisation reaction of the uridine derivatives, or the activation or transfer of sulfate. It was shown that the vitamin A-destroying enzyme lipoxidase can lower or abolish incorporation into mucopolysaccharide.

INTRODUCTION

The problem of the metabolic function of vitamin A, outside of the process of vision’ remains as yet unsolved. Observations described in previous papers of this series[1,2] pointed to an involvement of vitamin A in the biosynthesis of the glucocorticoid hormones. An entirely different approach to the problem was prompted by the results obtained by FELL *et al.*[3,4] and by FRAPE *et al.*[5]. The former showed that excess vitamin A, when added to the medium of bone cells grown in tissue culture, inhibited

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Abbreviations: MPS, mucopolysaccharides; CS, chondroitin sulfate; TCA, trichloroacetic acid; UDPGA, uridine diphosphate glucuronic acid; UDPAG, uridine diphosphate acetylglucosamine; UDPG, uridine diphosphate glucose; AG, acetylglucosamine; ATP, adenosine triphosphate; DPN, diphosphorpyridine nucleotide.

the synthesis of new cartilage and caused the transfer of sulfate groups bound in chondroitin sulfate to soluble sulfate containing compounds; and that excess vitamin A caused embryonic chick ectoderm to differentiate into mucus-secreting epithelium and inhibited keratinization. FRAPE and his group found that, ***in vivo***, vitamin A deficiency caused an increased sulfate uptake into connective tissue mucopolysaccharide, whereas a decrease was obtained when the vitamin was fed. These observations led us to the hypothesis that vitamin A, acting like a hormone, regulates the formation of mucopolysaccharides; excess vitamin A leading to an increase in the mucus type, a deficiency to an increase in the connective-tissue type.

A search was made for a mucus-forming tissue which could serve to test this hypothesis *in vitro*. Rat colon was found to be the most suitable, following the techniques developed by PASTERNAK *et al.*[6] for guinea pig colon. Using this system, it was possible to demonstrate that vitamin A has a direct function in the biosynthesis of colon mucopolysaccharide.

METHODS

Preparation of rats

For the preparation of vitamin A-deficient and pair-fed control rats, the strain, diet and techniques described by WOLF *et al.*[1] were used. The animals were deemed "deficient" at a time after feeding the deficient diet when weight loss had just set in[2].

Incubation of colon segments

After the rats were killed by decapitation, the colons were removed and cleaned of feces with ice-cold 0.9% saline. They were everted with the aid of a glass rod and cut into segments of about 1 cm long. The segments, weighing about 200 mg per incubation, were incubated with [^{35}S]sulfate (as [^{35}S]sodium sulfate, carrier free, from Abbott Laboratories) and 2 ml of Krebs–Ringer phosphate buffer, pH 7.3, containing glucose, as described by PASTERNAK *et al.*[6], at 37° for 4 h in a Dubnoff shaker in an atmosphere of 95% oxygen and 5% carbon dioxide. The [^{35}S]MPS were precipitated from the decanted medium at the end of the incubation by the addition of alcohol-benzene, as described below.

Incubation of colon homogenates

All steps were carried out at 4°. The rats were killed, the colons were removed and cleaned as described, and homogenized with 3.5 volumes of calcium and magnesium-free Krebs–Ringer phosphate buffer (0.01 M, pH 7.3), containing nicotinamide (0.3 M), for 2 min in a motor-driven, loose-fitting glass Potter-Elvehjem homogenizer. Debris and nuclei were removed by centrifugation at 600 × g for 15 min. The resulting supernatant suspension was used for incubation, by mixing with the co-factors and [^{35}S]sulfate or [^{14}C]glucose, described in the Tables to a final volume of 1 ml. The incubation was carried out at 37° for 2.5 h in an atmosphere of oxygen–carbon dioxide (95:5) in a Dubnoff shaker. The [^{35}S]MPS were isolated by the double precipitation method described below.

Isolation of mucopolysaccharides

The MPS formed during the incubations were isolated by two methods:

Single precipitation: After incubation, 9 volumes of alcohol–benzene (1:1, v/v)[7] were added to the incubation mixture, which was then left overnight at 4°, when a precipitate appeared. The mixture was centrifuged and the supernatant solution discarded. The precipitate was washed 3 times with the alcohol–benzene mixture and dissolved in 0.5 ml distilled water. To this was added 0.5 ml of an 8 % aqueous solution of TCA. The precipitated proteins were removed by centrifugation and the MPS in the supernatant solution was purified by descending paper chromatography. An aliquot of 0.2 ml of the solution was applied as a band to the baseline of a sheet of Whatman No. 1 filterpaper and developed in 1 *M* ammonium acetate–ethanol (2:5, v/v)[8]. MPS remained at the origin, whereas free sulfate moved with an R_F of 0.23. Similarly, during electrophoresis in barbital buffer at pH 8.8, MPS remained at the origin; in citrate buffer (0.05 *M*, pH 5.6), MPS moved 7 cm in 7 h (300 V).

Double precipitation: After precipitation and deproteinization as described above, the MPS were again precipitated from the remaining solution by addition of 9 volumes of the alcohol–benzene mixture, and left overnight at 4°. The precipitate was washed three times with alcohol–benzene and paper chromatographed as described.

Identification of mucopolysaccharides

The MPS were identified by paper chromatography and paper electrophoresis, as described above along with known samples of chondroitin sulfate, and agreement of the radioactive spots with the color spots given with the toluidine blue spray[9], was obtained.

Further, a sample of [^{14}C]MPS was hydrolyzed as described for CS by KORN[10] and the hexosamines isolated on a Dowex-50 column, along with carrier glucosamine, by the method of BOAS[11]. On paper chromatography, the spot of radioactive glucosamine coincided with the ninhydrin-color given by the carrier glucosamine.

A sample of the radioactive glucosamine was transformed into the osazone[12], along with carrier glucosamine, which was then recrystallized to constant specific activity.

A sample of [^{14}C]MPS (1.002 μC) isolated as described, was precipitated by cetylpyridinium bromide together with carrier CS (50 mg) (obtained from Nutritional Biochemical Corp.), as described by KORN[10], and the precipitate dialyzed, whereupon the radioactivity was retained in the non-dialyzable cetylpyridinium bromide complex of CS.

A further sample of [^{35}S]MPS, isolated as described, was hydrolyzed with 4 *N* hydrochloric acid at 100° for 2 h[8]. The hydrolysed solution was evaporated to dryness *in vacuo* over solid KOH and paper chromatographed in the ammonium acetate–ethanol system described above. All the radioactivity traveled with free sulfate (R_F 0.23) (R_F of methionine, 0.70; of cystine, 0.36)[8].

Vitamin A solution

Two mg of crystalline vitamin A were dissolved in 0.3 ml of absolute alcohol, followed by grinding of the solution with 1.7 ml of 0.2 % crystalline bovine plasma albumin in 0.1 *M* phosphate buffer, pH 7.4, in a glass homogenizer for approx. 2 min. The suspension was then centrifuged for 5 min at 500 × *g*. The supernatant solution was used after spectrophotometric assay of its vitamin A content. Vitamin A aldehyde and acid were dissolved similarly.

Protein determination

The protein content of the homogenate was determined by turbidity measurement. Suitable aliquots of homogenate were diluted to 3 ml with distilled water and mixed. To each tube, 3 ml 10% TCA were added, mixed and read after 10 min at 420 mμ in a Coleman colorimeter. A standard curve was made with crystalline bovine plasma albumin.

Estimation of hexosamine

The MPS formed, isolated by paper chromatography, was eluted from the paper with water. The eluate was evaporated to dryness in a stream of nitrogen on the steambath. To the dry residues, 1 ml of 3 *N* HCl was added and the MPS was hydrolyzed by refluxing for 4 h. Hexosamine was determined by the ELSON AND MORGAN procedure as modified by NEUHAUS AND LETZRING[13].

Measurement of radioactivity

A radioactivity scanning apparatus ("Actigraph", Nuclear Chicago Corporation) was used to determine ^{35}S and ^{14}C-activity on paperstrips. The paper chromatograms were passed beneath the counting tube of the scanner and peaks corresponding to radioactive spots were obtained on the chart of an automatic recorder. The paper under the peak corresponding to MPS was cut out and weighed. The weight of the paper was proportional to, and converted into, counts/min with the help of a calibration curve prepared by chromatographing and counting known quantities of [^{35}S]sulfate.

RESULTS

Effect of vitamin A-deficiency on the incorporation of [^{35}S]-sulfate into MPS

By rat colon segments: It was necessary to determine, whether there are differences between the synthesis of MPS in vitamin A deficient compared to normal rats. For this purpose, the colon segments from deficient and normal rats were prepared and incubated with [^{35}S]sulfate in Krebs–Ringer phosphate buffer, pH 7.4, as described. The MPS synthesized and secreted into the medium were isolated by the single precipitation method, purified by paper chromatography and counted by the scanning apparatus, as also previously described. The results are presented in Table I.

The incorporation of [^{35}S]sulfate into MPS by deficient colon segments was found to be about one-half compared to that of normal colons. When a suspension con-

TABLE I

INCORPORATION OF $^{35}SO_4^{=}$ INTO MUCOPOLYSACCHARIDES BY COLON SEGMENTS

The values are averages of the number of incubations shown in parentheses. Activity added, $19 \cdot 10^6$ counts/min; incubation period 4 h; —, deficient rat; +, normal.

Vitamin A status	*Mucopolysaccharide counts/min*
+	4247 (3)
—	1629 (3)

TABLE II

EFFECT OF ADDED VITAMIN A ON INCORPORATION OF $^{35}SO_4^=$ INTO MUCOPOLYSACCHARIDES BY COLON SEGMENTS

The values are averages of the number of incubations shown in parentheses. Incubation period 3.5 h, activity added $19 \cdot 10^6$ counts/min; +, normal rat; —, deficient rat.

Vitamin A status	*Vitamin A added*	*Mucopolysaccharide counts/min*
—	—	1279 (4)
—	10 μg (alcohol)	3515 (4)
—	10 μg (aldehyde)	3001 (4)
+	—	2685 (4)

TABLE III

INCORPORATION OF $^{35}SO_4^=$ INTO MUCOPOLYSACCHARIDES BY COLON HOMOGENATES

Each incubation contained $13.5 \cdot 10^6$ counts/min of activity and 16 mg of protein.

ATP 10 μmoles	*Mg++ 6 μmoles*	*Glutamine 1 μmole*	*DPN 3 μmoles*	*Glucose 10 μmoles*	*Mucopolysaccharide counts/min*
+	+	+	+	+	1,307
+	+	+	+	—	268
+	+	+	—	—	0
+	+	—	—	—	0
+	+	+	—	+	536
+	+	—	+	+	0
—	—	+	+	+	0
—	+	+	+	+	0

taining 10 μg of vitamin A was added to the incubation medium of the deficient colons, the incorporation was raised to the level of the normal (Table II). Both vitamin A and vitamin-A-aldehyde (retinene) were effective.

By rat colon homogenates: The conditions under which the incorporation of [^{35}S]-sulfate into MPS by rat-colon homogenates would take place, were first determined. The rat-colon homogenates, prepared as described, were mixed with the co-factors, as indicated in Table III, and incubated. The MPS were isolated by the double precipitation method, purified by paper chromatography and counted in the scanning apparatus as described. The incorporation was highest when the medium contained ATP, DPN, glucose and glutamine. When ATP or glutamine were omitted, no incorporation could be obtained, and on deletion of glucose or DPN, the incorporation was lowered. In all the experiments subsequently described, all the above co-factors were added to the incubation medium, and this will be referred to as "complete system".

Identification of MPS

Hexosamine contents, determined as described, of MPS before and after incubation were 3.8 μg and 17.4 μg per incubation, respectively, indicating net MPS-synthesis by rat-colon homogenates. Incubating [^{14}C]glucose, instead of [^{35}S]sulfate,

[^{14}C]mucopolysaccharide was isolated, presumably by formation of labeled hexosamine and glucuronic acid moieties of MPS.

As shown in Table IV, after hydrolysis of the [^{14}C]MPS obtained, the hexosamines, isolated by means of carrier glucosamine, retained constant radioactivity through recrystallizations of the osazone, as well as on paper chromatography. For further identification of the MPS, the toluidine spray of HAMERMAN[9] was used on the paper chromatograms and after paper electrophoresis, of the isolated MPS. A pink spot against a blue background, coinciding with the radioactive spot and with a CS spot, proved the presence of labeled MPS.

When [^{14}C]MPS, isolated in the usual manner, was mixed with carrier CS and precipitated with cetylpyridinium bromide, the complex thus formed retained radioactivity which, moreover, was not dialysable.

A sample of [^{35}S]MPS, when hydrolyzed under conditions leading to hydrolysis of the sulfate group only[8], yielded free radioactive sulfate. The same level of radioactivity incorporation was obtained whether incubation of the colon homogenate was carried out in presence or absence of chloromycetin.

Homogenates were then prepared from vitamin A-deficient and normal rat colons and incubated with the complete system. The results are summarized in Table V. The [^{35}S]sulfate incorporated into MPS by homogenates from deficient colons was once again about one-half that of normal colons. The addition of vitamin A-suspension to the deficient homogenates restored incorporation to normal. The suspending medium alone (serum albumin-ethanol) had no effect (Table V).

Specificity of vitamin A

To determine the specificity of vitamin A in reversing the decrease in the synthesis of MPS due to vitamin A-deficiency, suspensions of vitamins D, E and K_1 were added to the complete system of vitamin A-deficient colon homogenates. No fat-soluble vitamin except vitamin A could completely reverse the decrease in the synthesis of MPS. Vitamin E reversed partially. If vitamins E and A were added together, the increase in MPS synthesis went beyond that obtained with vitamin E alone. The results are shown in Table VI. Recently DOWLING AND WALD[14] have demonstrated that rats on a diet containing vitamin A-acid instead of vitamin A grew normally, although they developed night blindness. In our system also, vitamin A-acid, when added to the incubation medium of deficient homogenates, restored to normal the decrease in incorporation of [^{35}S]sulfate into MPS (Table VI).

Effect of addition of graded levels of vitamin A

It was of some interest to find out the effect of the addition of graded levels of vitamin A on restoring the decrease in the incorporation of [^{35}S]sulfate into MPS due to vitamin A-deficiency. The results in Table VII show that the maximum incorporation could be obtained when the amount of vitamin A added was 10–15 μg/6 mg of protein.

Experiments with hexosamines

The foregoing experiments have established that, under the conditions studied, the *in vitro* synthesis of MPS by rat colon is enhanced by vitamin A. The synthesis of MPS is complex and not all the steps are as yet known. The next object, therefore,

TABLE IV

INCORPORATION OF [^{14}C]GLUCOSE INTO HEXOSAMINES OF MPS SYNTHESIZED BY COLON HOMOGENATES

The complete system consisted of 10 μmoles ATP, 6 μmoles of $MgCl_2$, 1 μmole glutamine, 3 μmoles of DPN, 20 μmoles of Na_2SO_4 and 1,000,000 counts/min of [^{14}C]glucose

	Counts/min
Total [^{14}C]MPS isolated	15,200
Total [^{14}C]hexosamine obtained therefrom	6,232

Specific activity of hexosamine	*Counts/min/mmole*
After addition of carrier (54 μmoles)	5,000
After 2nd crystallization of osazone	6,800
After 3rd crystallization of osazone	6,750

TABLE V

INCORPORATION OF $^{35}SO_4^{=}$ INTO MUCOPOLYSACCHARIDES BY COLON HOMOGENATES

The values are averages of the number of incubations shown in parentheses. The complete system contained 10 μmoles of ATP, 6 μmoles of Mg^{++}, 1 μmole of glutamine, 3 μmoles of DPN and 10 μmoles of glucose — final volume 1 ml. Each incubation contained $10.6 \cdot 10^6$ counts/min of radioactive sulfate and 14 mg of protein. +, normal rat; —, deficient rat.

Vitamin A status	*Vitamin A added 10 µg*	*Mucopolysaccharide counts/min*
—	—	346 (3)
—	+	805 (2)
+	—	936 (4)
—	Serum-albumin-ethanol	268 (2)

TABLE VI

INCORPORATION OF $^{35}SO_4^{=}$ INTO MUCOPOLYSACCHARIDES BY COLON HOMOGENATES; SPECIFICITY OF VITAMIN A

The values are averages of the number of incubations, shown in parentheses. The complete system was that shown in Table V. Each incubation contained $9.78 \cdot 10^6$ counts/min of activity and 14 mg of protein.

Status of animal	*Addition (10 µg)*	*Mucopolysaccharide counts/min*
Normal	—	896 (2)
Deficient	—	483 (2)
Deficient	Vitamin A alcohol	841 (2)
Deficient	Vitamin A acid	805 (1)
Deficient	Vitamin D	232 (1)
Deficient	Vitamin E	608 (2)
Deficient	Vitamin E + vitamin A	752 (2)
Deficient	Vitamin K_1	435 (2)

TABLE VII

EFFECT OF ADDITION OF GRADED LEVELS OF VITAMIN A ON THE INCORPORATION OF $^{35}SO_4^=$ INTO MUCOPOLYSACCHARIDES

The values are averages of the number of incubations, shown in the parentheses. The complete system of Table V was used. Each incubation contained 10.6·10⁶ counts/min of activity and 16 mg of protein.

Vitamin A status	*Vitamin A added µg*	*Mucopolysaccharide counts/min*
Deficient	—	265 (2)
Deficient	5	409 (2)
Deficient	10	574 (2)
Deficient	15	584 (2)
Deficient	20	505 (2)
Deficient	30	571 (2)
Normal	—	562 (2)

TABLE VIII

EFFECT OF ADDITION OF HEXOSAMINES ON THE INCORPORATION OF $^{35}SO_4^=$ INTO MUCOPOLYSACCHARIDES BY COLON HOMOGENATES

The values are averages of the number of incubations, shown in parentheses. The complete system was that shown in Table V. In Expts. 2 and 3 glucosamine and galactosamine were substituted for glucose and glutamine. Each incubation contained 9.7·10⁶ counts/min and 14 mg of protein.

Vitamin A status	*Addition 11 µmoles*	*Mucopolysaccharide counts/min*
Deficient	—	250 (2)
Deficient	Glucosamine	215 (1)
Deficient	Galactosamine	233 (2)
Normal	—	562 (2)

was to localize the function of vitamin A in a specific step in the synthesis of MPS.

It was found that the vitamin is not involved in the formation of hexosamine, because glucosamine or galactosamine, when substituted for glucose and glutamine in the incubation medium of deficient homogenates, could not restore the decrease in incorporation (Table VIII).

Experiments with uridine diphosphate derivatives

Many workers have presented evidence that the UDP-derivatives are intermediates in the synthesis of MPS[15, 16]. It was decided, therefore, to find out whether vitamin A is required in a step before or after the involvement of UDP-derivatives.

At first, a system, requiring UDP-derivatives and capable of incorporating [^{35}S]-sulfate into MPS by rat-colon homogenates had to be developed. Table IX shows that the maximum incorporation of [^{35}S]sulfate in MPS occurs, when the incubation medium contained UDPAG, UDPGA, UDPG, AG, glutamine and ATP. This system shows an absolute requirement for glutamine, UDPAG and UDPGA, and partial requirement for UDPG and AG.

The system, containing UDP-derivatives, when incubated with deficient colon

TABLE IX

INCORPORATION OF $^{35}SO_4^{=}$ INTO MUCOPOLYSACCHARIDES BY COLON HOMOGENATES CONTAINING UDP-DERIVATIVES

Each incubation contained $8.5 \cdot 10^6$ counts/min and 14 mg protein. The complete system was that described in Table V, with the UDP-derivatives substituted for glucose.

UDPGA *1 µmole*	*UDPAG* *1 µmole*	*UDPG* *1 µmole*	*Glutamine* *1 µmole*	*AG* *8 µmoles*	*ATP* *1 µmole*	*Mg* *10 µmoles*	*Mucopolysaccharides* *counts/min*
+	+	+	+	+	+	+	895
+	+	+	+	+	+	+	770
—	+	+	+	+	+	+	0
+	—	+	+	+	+	+	0
+	+	—	+	+	+	+	286
+	+	+	—	+	+	+	0
+	+	+	+	—	+	+	376

TABLE X

EFFECT OF VITAMIN A ON THE INCORPORATION OF $^{35}SO_4^{=}$ INTO MUCOPOLYSACCHARIDES BY COLON HOMOGENATES CONTAINING UDP-DERIVATIVES

The values are averages of the number of incubations, shown in parentheses. The complete system consisted of 1 µmole of UDPGA, 1 µmole of UDPAG, 1 µmole of UDPG, 1 µmole of glutamine, 8 µmoles of AG, (all obtained from Sigma Chemical Corporation) 1 µmole of ATP and 10 µmoles of Mg^{++}. Each incubation contained $8.5 \cdot 10^6$ counts/min of activity and 14 mg of protein; +, normal rat; —, deficient rat.

Vitamin A status	*Addition of vitamin A 10 µg*	*Mucopolysaccharides counts/min*
+	—	832 (2)
—	—	270
—	+	681

TABLE XI

EFFECT OF LIPOXIDASE ON $^{35}SO_4^{=}$ INCORPORATION INTO MUCOPOLYSACCHARIDES BY COLON HOMOGENATES

Complete system was that shown in Table V. Each incubation contained $10.6 \cdot 10^6$ counts/min of activity and 14 mg of protein. Crystalline soybean lipoxidase was obtained from Nutritional Biochemical Corporation.

Preincubation for 1 h with lipoxidase (3 mg/ml)	*Addition of vitamin A 10 µg*	*Mucopolysaccharide counts/min*
0.01 ml	—	305
0.01 ml	+	538
0.04 ml	—	268
0.04 ml	+	0
0.07 ml	—	0
0.07 ml	+	0
0.10 ml	—	0
0.10 ml	+	0

homogenates, showed once again the lower incorporation of [^{35}S]sulfate into MPS (Table X). This decrease in the incorporation could be restored by the addition of vitamin A. It can, therefore, be inferred that vitamin A functions at some step beyond the synthesis of the UDP-derivatives.

Preincubation with lipoxidase

In an attempt to achieve independence from the use of vitamin A-deficient animals, and to find a means of destroying vitamin A *in vitro*, the colon homogenates were pre-incubated for 1 h with lipoxidase, an enzyme known to destroy vitamin A. This pre-incubation, as shown in Table XI, lowered or abolished the incorporation of [^{35}S]sulfate into MPS. However, addition of vitamin A restored incorporation only at low concentrations of lipoxidase, presumably because the added vitamin was destroyed by the excess lipoxidase present in the incubation medium.

DISCUSSION

Vitamin A-deficiency has for long been known to result in abnormal epithelial cells, leading to keratinization. Epithelia of mucus secreting cells, as for instance the salivary gland duct cells, are the first to be affected. Upon administration of vitamin A, these cells return to normal, both in morphology and function. In the work presently described, it could be shown that vitamin A is required for the synthesis of MPS of the type secreted by colon. It represents the first instance, apart from the reactions of the visual cycle, in which added vitamin A functions in a biochemical reaction sequence in a cell-free, *in vitro* system.

The assay used for MPS synthesis in the present work was the uptake of labeled sulfate from the medium into the final product of a many-step synthesis. Hence, the requirement of vitamin A for any particular step can only be derived by inference. For a more direct analysis, a dissection of the reaction sequence into constituent enzymic steps, and the influence of the vitamin thereon, is under way in this laboratory and will be the subject of a subsequent publication. In the meantime, it is possible to infer, albeit tentatively, from the present results, that the vitamin is not required for the following known steps: in the conversion of glucose to hexosamines; in the acetylation of hexosamines; in the conversion of hexosamines and glucose into their UDP derivatives; in the oxidation of UDPG to UDPGA. These conclusions could be reached because, starting with any of the above intermediates in place of glucose and glutamine, sulfate incorporation into MPS was still dependent on vitamin A. By a process of elimination, then, one could conclude that vitamin A functions either in the polymerisation of the UDP derivatives, or the activation or transfer of sulfate to the polymer. Confirmation of either of these alternatives must await the isolation of the separate reaction steps involved.

The very low level of incorporation (between 0.0022 and 0.025 %) of the added labeled sulfate needs some comment, since it might call into question the identity of the MPS produced. The following considerations support the contention that labeled MPS was synthesized: (a) The isolation procedure is designed specifically for MPS; (b) coincidence of a specific color reaction and radioactivity on paper after paper chromatography and paper electrophoresis in two solvent systems, and coincidence with the spot of carrier CS; (c) coincidence of ^{35}S and ^{14}C-radioactivity on paper chromatography and paper electrophoresis, after incubation with [^{35}S]sulfate and [^{14}C]glucose, respectively; (d) upon hydrolysis of the [^{14}C]MPS and isolation of the hexosamines with carrier glucosamine on a Dowex-50 column, the glucosamine carried radioactivity upon paper chromatography, as well as after conversion to, and recrystallization of, the osazone derivative; (e) the [^{14}C]MPS is precipitable, with carrier CS, by the specific MPS precipitation reagent, cetylpyridinium bromide. The precipitated complex retains radioactivity upon dialysis; (f) the [^{35}S]MPS can by hydrolyzed under conditions known to hydrolyze the sulfate group of MPS, producing free radioactive sulfate, identified by paper chromatography. Any sulfur-containing amino acids would have traveled at greatly different rates.

To exclude the possibility that the incorporation of labeled sulfate or glucose might be caused by bacterial infection of the homogenates, incubations were carried out in presence and absence of chloromycetin. No difference in incorporation was detectable.

The great differences in per cent incorporation of the added labeled sulfate from experiment to experiment requires some comment. The level of incorporation of labeled sulfate into colon MPS depends on the nutritional state of the animal. Although care was taken to use rats only at the point in the progress of their deficiency at which the weight-curve began to level off, the actual weights and nutritional states differed in each experiment. The animals for each separate experiment, on the other hand, were litter mates, and the deficient and normal were pair-fed, to ensure equality in weight, the only difference being due to the deficiency. Hence, each experiment is self-consistent, with its own normal controls, with which the results obtained from its own deficient animals should be compared.

That vitamin A is required in MPS synthesis could not be claimed unless evidence were obtained that the vitamin is present in colon mucosa. In fact, the unsaponifiable fraction of pig colon mucosa gave a positive Carr-Price reaction, indicating 3.7 μg of vitamin A/100 g of mucosa.

In view of the recent results of DOWLING AND WALD[14], it is of interest to note that vitamin A-acid as well as vitamin A and vitamin A-aldehyde are active in the present system, whereas other fat-soluble compounds are inactive. Using graded levels of vitamin A, a maximum response was obtained. Moreover, the vitamin A-destroying enzyme lipoxidase lowered or abolished MPS synthesis.

In order to achieve independence of the use of vitamin A-deficient animals, attempts were made to lower mucosal activity by isoctane extraction, by irradiation at 326 mμ, and by oxidation. In all three methods, sulfate incorporation by mucosal homogenate was lowered and could be raised to normal after addition of vitamin A. However, this was found to be non-specific, since vitamins E and K_1, and cholesterol achieved the same result.

In a different series of experiments carried out in this laboratory[17], it was found that corticosterone production by rat adrenal cortex *in vitro* was lowered in vitamin A-deficiency, and restored to normal by addition of TPNH (or TPNH generators such as glucose-6-phosphate or glycogen). In order to obtain a link between these, so far disconnected, functions of vitamin A, the above co-factors were added to deficient colon homogenate incubations, but failed to restore lowered MPS synthesis to normal.

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STUDIES ON MUCOPROTEINS

III. THE ACCESSIBILITY TO TRYPSIN OF THE SUSCEPTIBLE BONDS IN OVINE SUBMAXILLARY GLAND MUCOPROTEIN

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SUMMARY

Ovine submaxillary gland mucoprotein was found to be susceptible to trypsin. The evidence is a marked increase in terminal NH_2-groups and a corresponding rise in filterability through cellulose acetate membranes and an increase in electrophoretic mobility after trypsin treatment. Enzymic removal of the terminal neuraminic acid units of the prosthetic groups (6-α-D-N-acetylneuraminyl (2 ⟶ 6) N-acetylgalactosamine) increased the trypsin effect on ovine submaxillary gland mucoprotein by about 45 %. This means that at least one-third of the trypsin-susceptible bonds in ovine submaxillary gland mucoprotein are inaccessible to the enzyme molecule. This figure may represent only a minimum value since any steric hindrance exerted by the remaining N-acetylgalactosamine unit cannot be assessed at present.

The complete loss of virus haemagglutinin inhibitory capacity of ovine submaxillary gland mucoprotein on trypsin action is interpreted in terms of a critical molecular size below which a mucoprotein ceases to act as an inhibitor.

INTRODUCTION

In the first paper[1] of this series it was shown that the prosthetic group of OSM is the disaccharide α-D-N-acetylneuraminyl (2 ⟶ 6) N-acetylgalactosamine. OSM contains 58 % protein and 42 % carbohydrate. From the quantitative amino acid analysis[2] an average molecular weight of 100 for the component amino acids (referred to the amino acid residue) may be calculated. On this basis one in 6.4 amino acid residues carries a prosthetic group. The amino acid analysis revealed further that about one in sixteen amino acids is a diamino-monocarboxylic acid; this means that on the average there are 2.5 prosthetic groups to one diamino acid residue. It is known that trypsin cleaves specifically the peptide bond at the carboxyl end of lysine and arginine residues in the polypeptide chain. It was of interest, therefore, to investigate the effect on trypsin action of the prosthetic groups which actually are bulky substituents at the side chains of frequently occurring amino acids. As a test object for studying effects of steric hindrance on proteolytic enzymes OSM offers the advantage

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Abbreviations: OSM, ovine submaxillary gland mucoprotein; RDE, *Vibrio cholerae* neuraminidase; NANA, N-acetylneuraminic acid; BSM, bovine submaxillary gland mucoprotein.

that 60 % of the bulk of each individual prosthetic group can be specifically removed by *Vibrio cholerae* neuraminidase[1,3].

EXPERIMENTAL

Materials

OSM was prepared according to GRAHAM AND GOTTSCHALK[1].

Trypsin, twice recrystallized, was a Sigma preparation.

Crystalline RDE was prepared and kindly supplied by Dr. G. L. ADA, The Walter and Eliza Hall Institute, Melbourne.

Indicator viruses were prepared according to standard procedure[4].

Graded cellulose acetate filters were obtained from Membranfilter Gesellschaft Göttingen.

Methods

NANA was determined according to SVENNERHOLM[5] using NANA as standard. Readings were made in a Beckman Quartz Spectrophotometer Model DU (1.0-cm cells).

Quantitative ninhydrin determinations were carried out by the improved method of MOORE AND STEIN[6] using L-leucine as standard.

For the filtration tests the moistened membranes were placed on a sintered glass disc of a filter assembly and compressed between rubber washers. The free area of the membranes was 2.0 cm^2, and a constant pressure of 40 cm water was applied. 10 ml of the assay was placed on the membrane and the time required for the first 5 ml to pass through was recorded. The dilution of the filtrate due to the water content of the membrane was negligible.

Haemagglutinin inhibitory titrations were performed as described previously[1].

RESULTS

Number of peptide bonds split by trypsin

Action of trypsin on native OSM. OSM-trypsin: 42.5 mg OSM was dissolved in 10.0 ml 0.1 *M* phosphate buffer (pH 8.0) and 1.02 mg crystalline trypsin and a drop of toluene added. OSM-blank: prepared as assay, but omitting trypsin. Trypsin-blank: prepared as assay, but omitting OSM.

The three samples were kept for 5 h at 35° under gentle shaking. At the end of the incubation period the samples were appropriately diluted and their ninhydrin values determined. The experimental readings and their evaluation are given in Table I. The results show that by action of trypsin on 42.5 mg OSM under above conditions 9.63 mmole new N-terminals have been formed or 22.66 mmole/100 mg OSM as compared with 29.3 mmole/100 mg OSM to be expected from the amino acid analysis[2] of OSM, had all susceptible bonds been cloven (see note, Table II).

Action of trypsin on OSM pretreated with neuraminidase (RDE): 20.0 mg OSM was dissolved in 5.0 ml distilled water and the pH adjusted to 6.5 with a drop of 0.1 *N* NaOH; 2 drops of 1 % $CaCl_2$ solution were added.

0.97 mg crystalline trypsin was dissolved in 5.0 ml 0.2 *M* phosphate buffer (pH 8.0). OSM-blank: 1.0 ml of OSM solution plus 1.0 ml 0.2 *M* phosphate buffer.

TABLE I

N-TERMINALS LIBERATED BY TRYPSIN ACTION ON OSM

	Experimental values			*Evaluation of experimental values*	
		Extinction			
	Dilution for ninhydrin test	*Reading**	*Expressed as N-terminals mM*	*N-Terminals in total volume of assay mM*	*N-Terminals liberated by trypsin per 100 mg OSM mM*
OSM–trypsin	1:10	0.502	0.168	16.80	22.66
OSM–blank	1:5	0.389	0.131	6.55	—
Trypsin–blank	1:1	0.186	0.062	0.62	—
0.2 m*M* leucine standard	1:1	0.596	0.200	—	—

* Corrected for reagent blank.

Trypsin-blank: 1.0 ml trypsin solution plus 1.0 ml distilled water. OSM-trypsin: 1.0 ml OSM solution and 1.0 ml trypsin solution (see below). OSM-RDE-trypsin: To 1.0 ml of OSM solution was added 30,000 units RDE and the assay kept for 18 h at 35°. Then 1.0 ml trypsin solution was added and the digest kept for another 6 h at 35°.

The OSM-blank was kept for 24 h at 35°, the trypsin-blank for 6 h at 35°, the OSM portion of the OSM-trypsin assay for 18 h at 35° and after addition of trypsin solution for another 6 h at 35°. All samples contained 0.1 % toluene. At the end of the trypsin digestion the samples were analysed as above. The results are shown in Table II.

TABLE II

N-TERMINALS LIBERATED BY TRYPSIN ACTION ON NATIVE OSM AND OSM PRETREATED WITH NEURAMINIDASE

	Experimental values			*Evaluation of experimental values*	
		Extinction			
	Dilution for ninhydrin test	*Reading**	*Expressed as N-terminals mM*	*N-terminals in total volume of assay mM*	*N-terminals liberated by trypsin per 100 mg OSM mM*
OSM–trypsin	1:5	0.489	0.164	1.640	25.10
OSM–RDE–trypsin	1:10	0.297	0.099	1.980	33.60
OSM–blank	1:2	0.385	0.129	0.516	—
Trypsin–blank	1:1	0.178	0.060	0.120	—
0.2 m*M* L-leucine standard	1:1	0.597	0.200	—	—

Note: On calculating N-terminals it is assumed that ninhydrin colour intensities of peptides and standard are the same mole for mole. This assumption may underestimate peptides somewhat[6]; yet, comparison of such figures will scarcely be biased as systematic errors tend to cancel.
* Corrected for reagent blank.

After treatment of OSM with RDE which was shown previously[1] to split off 80 % of the total NANA, a subsequent digestion with trypsin increased the newly formed N-terminals by 33.9 % when compared with trypsin action on native OSM. If a correction is made for the 20 % NANA remaining after RDE treatment, though the relationship may not be strictly linear, the increase will amount to 42.4 %. In a similar experiment an actual increase of 37.8 % or a corrected increase of 47.2 % was found.

Physical changes following trypsin action on OSM

Filtration experiments. OSM-assay: 50 mg OSM was dissolved in 10.0 ml 0.1 *M* phosphate buffer (pH 8.0). OSM-trypsin assay: the same as above but with addition of 1.0 mg crystalline trypsin.

Both assays, after addition of a drop of toluene, were kept at 35° for 18 h with gentle shaking. The samples were then filtered through the cellulose acetate membranes (see METHODS) and the filtrates analysed for NANA. Table III shows that at 120 mμ average pore diameter the trypsin treated OSM passed the membrane with an efficiency exceeding 440 times that of native OSM. Even at 200 mμ, a pore size not significantly retaining the OSM-trypsin digest, the filtration efficiency of the native OSM was only 0.01 (or less) of that of the trypsin treated material.

TABLE III

FILTRATION RATES OF NATIVE AND TRYPSIN TREATED OSM

Assay	*Filter average pore diameter* mμ	*50 % filtration time min*	*NANA concentration as % of original*	*Filtration efficiency* × 10²*
OSM	120	$\geqslant$ 300**	44.4**	0.116
OSM	200	92.6	69.6	0.79
OSM	300	9.69	79.7	6.68
OSM–trypsin	120	2.59	83.3	51.1
OSM–trypsin	200	1.14	Not analysed	
Phosphate buffer	120	1.54		
Phosphate buffer	200	1.05		
Phosphate buffer	300	0.81		

* Calculated according to the formula

$$\frac{\text{50 \% filtration time of solvent}}{\text{50 \% filtration time of assay}} \times \frac{\text{NANA concentration of filtrate}}{\text{NANA concentration of input}}$$

** 1.6 ml passed the filter in 265 min; NANA determination was carried out on this aliquot.

Dialysis through cellophane: When an OSM solution (concentration range 0.5–1.0 %) was dialysed at 2° exhaustively against 10 volumes of distilled water and the dialysate lyophilized, analysis of its NANA content and dry weight determinations showed that less than 0.05 % of the mucoprotein escaped from the cellophane bag (cellulose casing of circumference 4.7 cm, Visking Corporation). In corresponding experiments with trypsin-treated OSM (0.25–1.0 % OSM in 0.1 *M* phosphate buffer, pH 8.0, and crystalline trypsin in 2 % concentration referred to OSM dry weight, 18 h at 36° in the presence of 0.1 % toluene) 0.7–1.6 % of the total NANA appeared in the dialysate.

15.0 ml of 0.6 % aqueous OSM solution (pH adjusted to 6.5) was pretreated with 60,000 RDE units in the presence of 0.1 % $CaCl_2$ and toluene at 35° for 18 h. After exhaustive dialysis and addition of an equal volume of 0.2 *M* phosphate buffer (pH 8.0) the mixture was digested with trypsin as above and dialysed against 10 volumes of distilled water. 45 % of the trypsin treated material was recovered in the dialysate. Of 90 mg casein digested with crystalline trypsin under similar conditions, 51 % were rendered dialysable.

Paper electrophoresis: 200 mg OSM was dissolved in 10 ml 0.1 M ammonium acetate–NH_4OH buffer, pH 8.6. 0.5 ml of above solution was taken as OSM solution. To 9.5 ml of above solution was added 4.0 mg crystalline trypsin. Both assays were kept at 35° for 18 h. At the end of the digestion period 50 μl of each assay was applied to Whatman No. 4 paper strips (10 × 5 cm), previously equilibrated against the buffer. Electrophoresis was carried out for 8 h at 20° in a potential gradient of 10 V/cm at 18 mA. After 8 h the strips were dried at 80° and cut across their width into 5 mm segments. Each of these and a paper-blank were submitted to the procedure of NANA determination. The results in terms of extinction are given in Fig. 1.

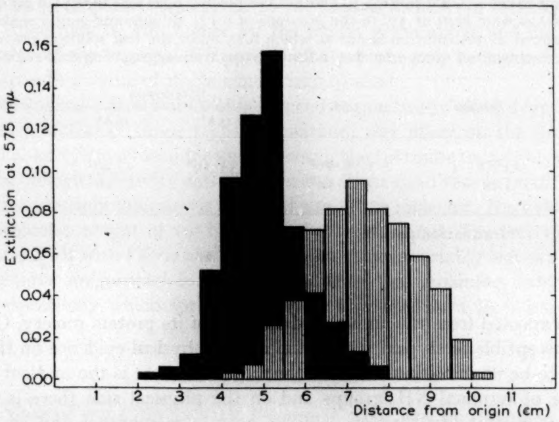

Fig. 1. Paper electrophoresis of native (black columns) and trypsin-treated (hatched columns) OSM.

Evaluation of the data presented in Fig. 1 shows that the areas under the two curves fall within 2 % of each other. The average mobility of native OSM is $1.67 \cdot 10^{-5}$ cm^2/V/sec and that of trypsin-treated OSM $2.39 \cdot 10^{-5}$ cm^2/V/sec. The distribution of the patterns does not differ significantly from the normal distribution. The standard deviations of the curves are $\pm$ 0.31 and $\pm$ $0.45 \cdot 10^{-5}$ cm^2/V/sec respectively, *i.e.* the scatter of the OSM–trypsin curve is 45 % greater than that of the OSM curve. In a similar experiment on cellulose acetate strips the mobility of the OSM–trypsin assay approximated that found on paper strips, whereas native OSM did not move from the site of application.

Loss of biological activity of OSM on trypsin action

OSM is the most potent influenza virus haemagglutinin inhibitor prepared so far. Its inhibitory titres against two different strains of influenza virus in the indicator state before and after trypsin action are shown in Table IV. As may be seen, the inhibitory titres of OSM against PR8 and Lee indicator viruses were reduced after 16 h digestion with trypsin 88,000 and 15,500 times respectively. The reduction in titre was accompanied by a complete loss of viscosity; no measurable amounts of free NANA were present in the lyophilized dialysate of the OSM–trypsin assay. In this context it may be mentioned that the inhibitory titres of OSM against the same indicator viruses were reduced after exhaustive treatment with *Vibrio cholerae* neuraminidase only 172 and 53 times respectively; in this case the reduction in titre was accompanied by the release of an average of 80 % of the total NANA content (GRAHAM AND GOTTSCHALK[1]) and also by a considerable decrease in viscosity (GOTTSCHALK AND THOMAS[7]).

TABLE IV

INHIBITORY TITRES PER MILLIGRAM OSM AND THEIR REDUCTION BY TRYPSIN

Conditions of test: 20 mg OSM was dissolved in 15.0 ml 0.1 *M* phosphate buffer (pH 8.0). 2.0 ml of the solution was taken as OSM control, to another sample of 2.0 ml was added 1.0 mg crystalline trypsin. Both assays were kept at 35° in the presence of 0.1 % toluene and gently shaken. Inhibitory titre is defined as the dilution factor at which 0.25 ml of the test solution prevents 3 out of 4 haemagglutinating doses of a particular indicator virus from aggregating fowl red blood cells.

Indicator	*OSM*	*OSM-trypsin*	
		*0.25 h**	*16 h**
PR8 – i	$1.76 \cdot 10^6$	$5.35 \cdot 10^2$	< 20
Lee – i	$3.11 \cdot 10^5$	$1.65 \cdot 10^2$	< 20

* Incubation period.

DISCUSSION

As was to be expected from the amino acid analysis of its protein moiety, OSM was found to be susceptible to trypsin. The chemical and physical evidence on this point would appear to be unambiguous. On the chemical side there is the marked increase in the number of terminal NH_2-groups and on the physical side there is a corresponding rise in filterability through cellulose acetate membranes and an increase in electrophoretic mobility after trypsin digestion, although the overall net negative charge of OSM at pH 8.6 cannot rise by more than 10 % on splitting the susceptible bonds. These results would indicate fragmentation of the native OSM molecule on trypsin action.

The extent to which these fragments separate cannot be accurately assessed on these data alone. Thus it is conceivable that fragments are held together by linkages other than peptide bonds; and the physical methods applied provide only compound measures of molecular weight and axial ratio. That in fact reduction in molecular weight is the major contributor to the observed physical effect of trypsin on OSM is proven directly by independent evidence. Work in progress by Mr. M. B. SMITH, Physical Chemistry Unit, Division of Food Preservation, C.S.I.R.O., Sydney, clearly shows that the molecular weight of native OSM (about $1 \cdot 10^5$) is greatly reduced on trypsin digestion (to about $9 \cdot 10^3$). The latter figure also accounts for the very small degree of dialysability (through cellophane) of the OSM–trypsin digest.

The main outcome of the present work is the demonstration that the multiple prosthetic groups distributed along the polypeptide chain provide steric hindrance for the close approach of the active centre of trypsin to the potentially susceptible bonds. The finding that after enzymic removal of the terminal NANA unit of the prosthetic group the trypsin effect on OSM increased by about 45 % would indicate that at least one-third of the susceptible peptide bonds are inaccessible to the enzyme molecule because of the screening effect of the prosthetic groups. It is quite possible that an even larger proportion of the susceptible bonds is inaccessible to trypsin since the remaining unit (N-acetylgalactosamine) of the prosthetic group may still exert some steric hindrance. The reduction in size of the prosthetic group and the more extensive action of trypsin after pretreatment of OSM with neuraminidase render the products of trypsin digestion dialysable through cellophane.

It is an inference from these data that the diamino-monocarboxylic acid content of OSM, as reported earlier[2], is too low. On the assumption that after removal of NANA all susceptible bonds are split by trypsin the total diamino acid content is higher by 12 % than the value given by the MOORE AND STEIN technique. However, the true value may be still higher for the reason given above and because the ninhydrin value of the terminal NH_2-group of a peptide may be somewhat lower than the corresponding value of the leucine standard used.

The biological activity of OSM is reduced to practically nil by trypsin, as already observed by MCCREA[8]. Since trypsin is without any effect on the linkages joining NANA to N-acetylgalactosamine and N-acetylgalactosamine to the polypeptide chain, this loss in biological activity can only mean that one condition to qualify as influenza virus haemagglutinin inhibitor is a critical size of the molecule. The evidence suggests that a molecular weight of 10^4 is already below this limit. The fact that treatment of OSM and BSM with *Vibrio cholerae* neuraminidase invariably leaves a residual titre of considerable magnitude[1] is apparently due to the remaining 20 % and 28 % of NANA respectively which for reasons to be discussed in a later paper cannot be removed.

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CHANGES DURING THE DIVISION CYCLE IN BACTERIAL CELL WALL SYNTHESIS, VOLUME, AND ABILITY TO CONCENTRATE FREE AMINO ACIDS

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SUMMARY

Changes in cell volume, cell wall synthesis, and ability to concentrate reversibly an amino acid were studied in *Alcaligenes fecalis*. Cell volume and number were measured in a Coulter Counter; cell wall synthesis was followed by means of short-term incorporation of radioactive alanine and methionine; the ability of the cell to concentrate replaceable methionine served as a measure of the concentration of an amino acid. All of these parameters were studied in synchronously dividing cultures. In addition, cell volume and methionine concentration were studied in spheroplasts and in cells leaving the lag and entering the exponential phase of growth.

Whereas cell wall synthesis was found to occur at an increasing rate throughout the cell division cycle, cell volume remained constant, except for a rapid increase just before division. The maximum amount of methionine which could be concentrated also remained constant throughout most of the division cycle. In spheroplasts an increase in volume was not accompanied by an increase in ability to concentrate methionine. Cells leaving the lag phase of growth showed an increased ability to concentrate methionine not accompanied by a comparable increase in cell volume.

The results indicate that there is no direct relationship between cell volume, ability to concentrate methionine, and cell wall synthesis. However, cell volume and the concentration of replaceable methionine are regulated in conjunction with the division cycle.

INTRODUCTION

In bacteria, the cell wall is responsible for the shape of the cell and forms the barrier separating the interior of the cell from the environment. Little is known about the synthesis of cell wall material during the cell division cycle. Similarly, changes in those properties of the cell which may be expected to be related to cell wall synthesis, such as cell volume or the ability of the cell to concentrate medium metabolites reversibly, have not been studied with reference to division, except that SCOTT

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* A portion of this work will be submitted by Cynthia Lark to the Graduate School of Saint Louis University in partial fulfillment of the degree of Doctor of Philosophy. This portion of the work is printed here by permission of the Graduate School of Saint Louis University.

AND CHU[1] have obtained results which suggest that the ability of *E. coli* to concentrate glucose reversibly may vary during the division cycle.

During the past two years methods for purification and analysis of bacterial cell wall material have been improved[2–4]. Recent advances in particle sizing[5,6] and in studies on metabolite transport in bacteria[7,8] have made it possible to study the variation in these cell parameters with greater accuracy and technical ease.

We have undertaken to determine when, during the division cycle, the bacterial cell wall is synthesized and whether synthesis of this material controls cell volume or the capacity of the cell to concentrate a medium metabolite.

The organism used in this study was *Alcaligenes fecalis*. A method for synchronizing the division cycles of the individual cells of a culture[9,10] has been described previously. The cell wall of this organism has been shown to consist of two fractions, one soluble in phenol, the other insoluble[11]. The latter fraction is responsible for the rigid rod-shape of the organism and is characterized by the presence of a few amino acids in extremely high concentration, the principal one being alanine.

MATERIALS AND METHODS

Growth and synchronization of A. fecalis *Strain LB*

The properties of Strain LB of *A. fecalis* and the materials and methods used for its growth and maintenance have been described[10,12,13].

Synchronization of cell division was carried out by a modification of a method described previously[10]. This modification was designed in collaboration with Dr. Y. MARUYAMA in connection with work presented elsewhere[14]. 7 l cultures of cells were grown at 37° in the medium in which cell division was to be studied (casamino acid medium or L amino acid medium[13] lacking methionine and alanine) to a titer of $2 \cdot 10^8$ cells/ml. The bacteria were sedimented in a Servall continuous flow centrifuge at 30° at a speed of 10,000 rev./min, the culture being passed through the centrifuge at the maximum rate, *i.e.*, 4 min/l. The sediment was resuspended in 15 ml of nitrogen-free medium at 37° and homogenized for 45 sec in a Waring blendor. The resulting suspension was adsorbed on to the top layer of a filter paper pile consisting of nine pieces of Eaton-Dikeman paper (No. 624–63) and clamped in a plastic funnel (internal diameter 15 cm). It was then rapidly washed through the filter paper pile with 300 ml of nitrogen-free medium under suction*. Synchronized growth was initiated by eluting the middle three papers within 1 min into 300–400 ml of complete medium to start the experiment.

Measurement of cell wall synthesis

Cell wall synthesis was measured by means of DL-[^{14}C]alanine and DL-[^{35}S]-methionine. 10 ml of a bacterial suspension growing in alanine- and methionine-free medium were added to 120 μg of radioactive alanine or methionine, and incubated with aeration for 5 min in a stainless steel centrifuge tube. The incorporation was stopped by the addition of 0.1 ml of saturated [^{12}C]alanine or [^{32}S]methionine, and the mixture frozen within 30 sec in a dry ice–alcohol bath.

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* We have found that the amount of liquid to be used in washing must be determined empirically and may vary with the type of funnel used. In addition, it is important to make sure (using ink or a dye) that organisms are adsorbed only to that area of the top layer which will filter through to the bottom layers. Organisms should be eluted only from that area of the paper directly in the main flow of the wash fluid.

Subsequently, samples were thawed to 0°, and centrifuged twice at 40,000 × *g* for 60 min to remove medium activity. Heat killed bacteria were added to facilitate the centrifugation and fractionation. Cell wall and cytoplasmic protein were then fractionated as described previously[11]. By this method, the rate of incorporation of alanine or methionine into cell wall was measured. Cells which were labeled with [^{14}C]alanine or [^{35}S]methionine showed no loss of radioactive label from the cell wall or cytoplasmic fractions on subsequent incubation and growth in radioactive free medium containing 0.01 *M* [^{12}C]alanine or [^{32}S]methionine. This indicated that the rate of incorporation of alanine or methionine represented a synthetic rate and was not due to a non-conservative exchange or turnover reaction.

Measurement of methionine concentrating ability

As the concentration of replaceable methionine (pool methionine) in exponential cultures of *A. fecalis* had been studied in detail previously[13], methionine was used again in these studies. Because determinations had to be carried out rapidly, certain modifications were made in the procedure for measuring methionine accumulation. 10 ml samples of cultures were added to chloramphenicol (500 μg/ml) and radioactive DL-[^{35}S]methionine (12 μg/ml). The purpose of adding chloramphenicol was to stop protein synthesis and thus preserve the cells' internal pool of free methionine. After exactly 3 min, a 5-ml aliquot (A) of this mixture was filtered through a Millipore filter, size DA, to collect the bacteria. These were immediately washed by passing 5 ml of methionine-free medium through the filter. The total time for filtration and washing was 30 sec. (Experiments had shown that a wash filtration by methionine-free medium did not remove measurable quantities of pool methionine.) This 5-ml aliquot measured the total methionine concentrated by the cell in all forms. Simultaneously with the time aliquot A was filtered, 5 mg of non-radioactive methionine was added to the remaining 5 ml of culture (B). This was incubated for 3 more minutes and then filtered and washed as the first. Sample B measured non-replaceable methionine within the cell. The difference between A and B measured the replaceable methionine concentrated during the first 3 min period of incubation.

Previous work[13] showed that amounts of L-methionine in excess of 3 μg/ml saturated the cells' methionine concentrating system. The present method yielded consistent results in that medium concentrations of 12 μg, 24 μg, or 6 μg/ml DL-methionine all yielded the same total amount of methionine replaceably accumulated by the cell.

Measurement of cell volume

Cell volume was measured by means of the Coulter Particle Counter, Model A (Manufactured by Coulter Electronics, Chicago, Ill. (U.S.A.)). The theory of operation of this machine, as well as a trial application to bacterial counting and sizing, have been described[5,6].

In the experiments to be reported, we used a 27-μ diameter orifice supplied by the Coulter Electronic Company. Maximum gain was used to obtain a maximum range on the sizing scale.

The counting medium was isotonic sodium chloride (Abbott Laboratories), which gives a low background due to absence of countable particles. (This background = 1000–1200 particles of size greater than 0.13 μ^3 in 0.05 ml). Samples were diluted either directly in saline for counting, or following fixation in an 0.2% formalin solution. Formalin fixation preserved the samples for counting and split a large proportion of double cells. Samples for counting were usually diluted in a volume of 25 ml of saline at room temperature in such a manner as to give 20,000 particles in 0.05 ml of a size of 0.2 μ^3 or greater. (In this size range, the background from a comparable blank lacking only bacteria was approx. 700 particles/0.05 ml). At this bacterial concentration no appreciable coincidence effect was observed. This was checked by diluting the sample and ascertaining that the volume/count ratio was constant. This ratio would be increased by an appreciable coincidence effect.

Counts were carried out at a current setting of six on the machine, which yielded a maximum pulse height per particle without causing boiling in the orifice due to heating. The machine was calibrated with polystyrene latex particles (Dow Company) with a volume of 0.84 μ^3.

Particle volume was determined by counting the number of particles in a given size range, multiplying the number of particles in a given size range by the average volume of this range, and adding the products for the various size ranges. This sum gave the total cell volume in the 0.05 ml counted. Division by the total cell count yielded the average volume/cell.

The results obtained with the Coulter Counter were compared with those obtained by centrifuging the cell suspension in a hematocrit tube and visually calculating the volume. Values for bacteria obtained from a stationary phase culture of *A. fecalis* (presumably small cells) and from one leaving the lag phase (presumably larger cells) are shown in Table I. Despite the differences in absolute values, the volume ratios of the two cell types agree well. The difference in absolute volume can be expected, since the Coulter Counter measures only the volume of electrolyte displaced by an isolated cell, whereas the hematocrit method includes intercellular water as well. (This latter factor can be quite large, since the rod-shaped bacteria will not necessarily pack into a closely packed parallel arrangement, but rather in a randomly oriented fashion.) As a result of this experiment, it seemed safe to conclude that measurements of relative cell volume could be made with the Coulter Counter.

TABLE I

RELATIVE VOLUME MEASUREMENT BY HEMATOCRIT AND BY COULTER COUNTER

	Volume/cell	
	Hematocrit	*Coulter counter*
(A) Stationary phase cells	0.744 μ^3	0.33 μ^3
(B) Late lag phase cells	1.05 μ^3	0.50 μ^3
A/B	0.708	0.660

RESULTS

The rate of cell wall synthesis during the division cycle of A. fecalis

Aliquots were removed at intervals from a synchronized culture and tested for rate of synthesis of cell wall as indicated by the incorporation of [^{14}C]alanine or

[^{35}S]methionine. As may be seen in Fig. 1, the rate of synthesis of both the phenol-soluble and phenol-insoluble cell wall components, as well as of cytoplasmic protein, increased steadily during the division and the inter-division periods. At no time was there a sufficiently large decrease in the synthetic rate to indicate a cessation of cell wall synthesis during the cell division cycle*.

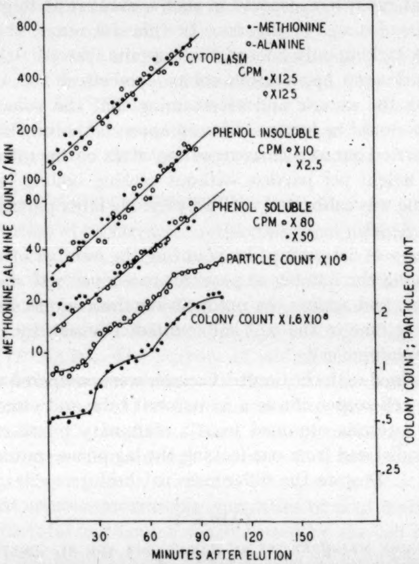

Fig. 1. Cell wall synthesis in synchronized cultures of *Alcaligenes fecalis*. Cell wall synthesis measured as counts/min of alanine or methionine incorporated into the cell. For the procedures used in carrying out these experiments see MATERIALS AND METHODS. The CPM of the various fractions have been normalized for convenience of presentation. To obtain the absolute values, they must be multiplied by the factors shown. The specific activity of the [^{14}C]alanine used was 4 mC/mmole, that of the [^{35}S]methionine, 30 mC/mmole.

The data in Fig. 1 afford a comparison of counts of the number of cells by means of colony count and by the particle counter. From these data, as well as those published previously[15], we have concluded that colony formers plate with an efficiency of about 70–80 % of the particle count.

Concentration of pool methionine during the cell division cycle

Fig. 2 shows the results of an experiment in which aliquots taken at intervals from a synchronized culture were tested for their methionine concentrating ability. As may be seen, the amount of pool methionine found at any time in the culture (under conditions of pool saturation), paralleled very closely the cell count. This amount was equal to that found in exponentially dividing cultures and corresponded to about $5 \cdot 10^5$ molecules per cell*. The finding that the concentration of pool methionine per cell was constant during the cell division cycle implied that the duplication of the responsible mechanism might be closely synchronized with the separation of the cell into two daughter units.

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* Because of the scatter of the points, it was impossible to ascertain whether minor periodic variations in the rate of incorporation of [^{14}C]alanine into the cell wall fractions may, in fact, occur. However, if this were so, such variations would be less than ± 30 % of the average rate.

Alternatively, some other attribute of the cell closely linked to cell division might limit the amount of pool methionine concentrated by the cell. The most obvious such parameter was cell volume.

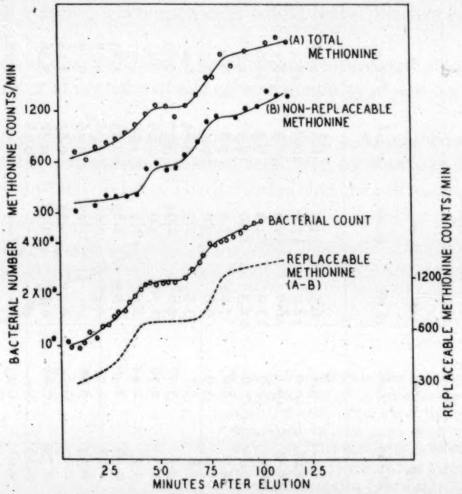

Fig. 2. Replaceable methionine concentration in synchronized cultures of *Alcaligenes fecalis*. Concentrated methionine is given in counts/min. For the conditions of the experiment see MATERIALS AND METHODS.

Changes in cell volume during the division cycle

Table II presents a volume analysis of cells taken at intervals from a synchronized culture. 0.1-ml samples were taken at the times indicated and added to 1 ml of 0.2 % formalin. During the 24-h period subsequent to the experiment, the samples were analyzed and sized. (Fixation in formalin was necessary because of the length of time required for the sizing analysis. Although it was possible that fixation itself might change cell volume, no further change in particle number, size distribution, or average cell volume occurred in cell suspensions during immersion in 0.2 % formalin for 24 or 48 h). Table II gives the number of cells at any time, the percent in any size range, and the average cell volume. A comparison of the average cell volume immediately before or after rapid increases in cell number, *i.e.*, at 20, 35, 60, and 70 min, revealed no major shift in cell volume or in distribution of cell size.

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* This value is lower than that found previously[13] for L-methionine. This is due in part to the higher efficiency of the Coulter counter. In addition, D-methionine might also be concentrated in place of L but to a lesser extent. Finally, the shorter period in chloramphenicol (3 min instead of 20 min) might result in a saturated pool of smaller size.

TABLE II

SIZE ANALYSIS OF CELLS TAKEN FROM A SYNCHRONIZED CULTURE

Samples were taken from a synchronized culture as described in the text, and fixed and sized as described in MATERIALS AND METHODS. Results from an exponential culture are given for comparison.

Minutes after elution sample was taken	*20*	*25*	*30*	*35*	*40*	*45*	*50*	*60*	*65*	*70*	*Non-synchronized exponential culture*
Total number greater than 0.32 cubic microns	*12,840*	*13,223*	*16,174*	*18,700*	*18,000*	*19,386*	*22,168*	*23,508*	*28,300*	*39,021*	*20,745*
% in size range (cubic microns) =											
0.32–0.4	2.7	4.2	3.0	4.4	3.3	6.1	3.5	6.6	6.05	6.5	1.8
0.4 –0.48	10.4	10.2	21.4	22.8	20.0	18.0	17.0	20.0	18.4	20.7	19.7
0.48–0.56	15.5	19.1	17.5	18.5	19.1	16.0	19.6	21.3	18.5	18.4	15.6
0.56–0.64	14.9	12.8	17.8	16.8	16.9	17.0	14.3	9.9	14.6	14.8	16.0
0.64–0.72	13.2	13.2	9.1	10.8	11.0	12.3	14.0	14.3	9.7	11.9	13.9
0.72–0.80	9.3	7.9	10.5	4.9	8.6	7.05	7.4	3.8	9.6	6.1	8.1
0.80–0.88	8.6	7.8	8.9	7.6	3.9	6.8	10.6	9.5	5.5	6.7	5.3
0.88–0.96	4.2	4.7	5.3	3.8	5.7	3.5	1.3	3.84	6.0	2.86	4.6
0.96–1.04	4.7	7.1	5.25	3.7	3.06	4.6	2.94	3.2	2.83	3.5	2.8
1.04–1.12	2.9	2.8	2.95	1.5	2.76	1.26	3.0	2.26	2.4	2.3	4.3
1.12–1.20	2.5	2.5	2.3	2.1	1.45	2.0	1.4	1.68	2.5	2.26	2.4
1.20–1.28	1.68	2.4	1.6	0.96	2.46	1.95	1.85	0.85	1.35	1.24	2.4
1.28–1.36	1.67	0.69	1.3	1.01	0.27	1.37	0.82	1.08	0.72	1.09	1.1
1.36–1.44	1.25	0.69	0.204	0.53	0.86	—	1.25	0.36	0.19	0.43	0.3
1.44–1.52	2.9	1.66	1.5	—	0.24	0.75	0.53	0.85	1.14	0.57	0.5
1.52–1.60	0.195	0.23	—	0.73	0.68	0.94	0.75	0.32	0.36	2.7	0.34
1.60–1.68	0.47	0.88	0.55	0.19	—	—	—	0.18	—	0.615	0.86
1.68–1.76	—	0.144	0.25	0.01	0.23	—	—	—	0.164	0.0206	—
Average volume in cubic microns	0.72	0.72	0.76	0.65	0.67	0.66	0.68	0.65	0.66	0.68	0.71

The data indicated that, during the division cycle, volume was essentially constant per cell unit. To check the possibility that this might be the result of formalin fixation itself, an experiment was carried out in which samples were analyzed without formalin fixation. Each measurement had to be completed in 1 min, because the bacteria were growing. The analysis was, therefore, limited to a single size category in which variation would most sensitively reflect changes in cell volume. The population chosen was that of smaller cells of size 0.35 μ^3 to 0.5 μ^3. This cell population could be increased by division of larger into smaller cells. It could be decreased by an increase in cell volume, whereupon cells would leave this size category to enter a larger one.

Samples taken at intervals from a synchronous culture were diluted directly into saline and the number of particles of size greater than 0.35 μ^3 and 0.5 μ^3 were counted within 1 min.

The results from this experiment are shown in Fig. 3. As may be seen, the number of smaller cells in the population remained relatively constant, as did the total cell count until the onset of cell division. This indicated that the cell maintained a constant volume during the majority of the period between successive divisions. Just before cell division, cell volume rapidly increased—as was indicated by a loss of cells from the smaller cell category. This finding supported the detailed analysis already presented in Table II.

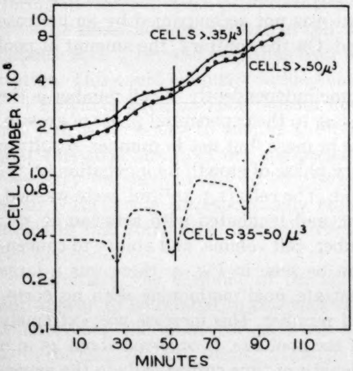

Fig. 3. Variation in the number of cells of volume 0.35 μ^3 to 0.5 μ^3 in synchronized cultures of *Alcaligenes fecalis*. The experimental procedures are described in the text and in MATERIALS AND METHODS. The percentage of cells in the 0.35–0.5 μ^3 size range is somewhat larger in cell suspension fixed in formalin (see Table II) due to a splitting of double cells by formalin. Cells were diluted at 45 and 90 min to maintain a constant density range. Dilutions have been corrected in plotting the results.

Relation of cell volume to pool methionine concentration

The experiments just described suggested that the maximum concentration of methionine which could be replaceably concentrated might be controlled by the cell volume. To test this, experiments were carried out in which cell volume was varied in dividing and non-dividing cells and the concentration of pool methionine was measured.

Cells which are treated with 50 units/ml penicillin enlarge into spheres (spheroplasts) whose size may increase greatly with time. In addition, they have lost most of the phenol-insoluble portions of their cell wall[11]. This increase in size parallels the

TABLE III

REPLACEABLE METHIONINE CONCENTRATION IN NORMAL CELLS AND SPHEROPLASTS

Cell number and volume measured with the Coulter Counter on formalin fixed cells. Replaceable methionine was measured as described in the MATERIALS AND METHODS.

	Normal cells	*Spheroplasts*
Cell number/5 ml	$4.05 \cdot 10^9$/ml	$1.35 \cdot 10^9$
Replaceable concentrated Methionine/5 ml	0.50 γ	0.16 γ
Average cell volume	0.69 μ^3	1.67 μ^3
Methionine/10^9 cells	0.125γ	0.119γ
Methionine/mm^3 cells	0.178γ	0.071γ

increase in protein per cell unit and spheroplasts may reach a volume of 10 times the normal cell volume[16].

An exponential culture of *A. fecalis* was divided into two aliquots, to one of which was added penicillin to a concentration of 50 units/ml. After 1 h the number of cells, cell volume, and maximum amount of methionine which could be replaceably concentrated, were measured in both aliquots. The results shown in Table III indicated that the increase in volume in the spheroplast was not accompanied by an increase in the amount of pool methionine accumulated. On the contrary, the amount of pool methionine remained constant per cell unit.

Another system for varying the cell volume independently of cell number is the transition of cells from the stationary, *via* the lag, to the exponential phase of growth. During the lag phase of growth, cells increase in mass, but not in number. A culture of *A. fecalis* was allowed to enter the stationary phase of growth by incubation at 37° in casamino acid medium with aeration for 36 h. The cells ($1.5 \cdot 10^{10}$/ml) were washed, diluted 1/50 into fresh L amino acid medium, and incubated with aeration at 37°. Samples were taken at intervals, and cell number, cell volume, and ability to concentrate pool methionine were measured. As can be seen in Fig. 4, there was a large initial increase in the cells' ability to concentrate pool methionine with no corresponding increase in either cell volume or cell number. This increase was extremely abrupt and amounted to almost a doubling of the pool size. It occurred about 45 min before the first increase in cell number. This length of time corresponds to the generation time of *A. fecalis* when it grows exponentially in this medium under these growth conditions.

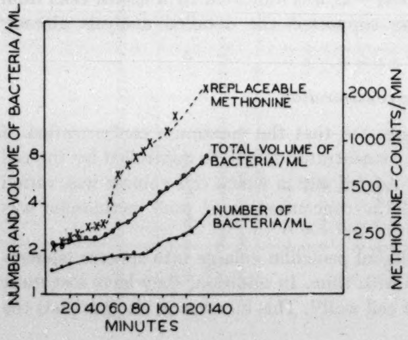

Fig. 4. Changes in replaceable methionine concentration and in cell volume of cells during the transition from the stationary to the exponential phase of growth. The methods used are identical with those used in the previous experiments. Relative cell volumes are of bacteria contained in 1 ml. Replaceable methionine was measured as in Fig. 2. Cells in the stationary phase of growth were diluted in to fresh medium at 0 min. Cell number and volume ordinate: Number of bacteria × ($2 \cdot 10^8$). Volume of bacteria × ($6.25 \cdot 10^{-2}$ mm^3).

From the experiments in Table III and Fig. 4, it is obvious that an increase in cell volume is neither a sufficient nor a necessary condition for an increase in the ability of the cell to concentrate pool methionine.

DISCUSSION

In the experiments described above we have studied the variation in three parameters of cell growth during the cell division cycle. Two of these, volume and ability to concentrate pool metabolites (pool methionine), remain constant per cell unit, because they increase simultaneously with cell division. The third, synthesis of cell wall material, proceeds continuously during the period between cell divisions as well as during the division period.

Microscopic examination during this interval between successive divisions shows that cells become elongated. Because they do not increase in volume, it must be assumed that they become thinner and that the surface/volume ratio must increase*. Thus, it is not necessary to assume that continued synthesis of cell wall material during the inter-division period leads to a thicker cell wall.

The simultaneous increase in cell volume and in pool metabolite capacity may balance each other and may thus lead to a constant intracellular metabolite concentration. This could eliminate sudden changes in internal osmotic pressure.

Previous work on spheroplasts has indicated that the presence of a bacterial cell wall is necessary to confine the cell volume. The present work would indicate that this structure does not completely control cell volume, though its presence is necessary for such control to be exercised. Changes in cell volume during division occur without any correspondingly radical change in rate of cell wall synthesis. This indicates that some other control over cell volume exists, which is closely related to cell division.

Similarly, the doubling of the cell's capacity to concentrate metabolites does not appear to be related to cell wall synthesis, but rather to cell division. Thus, in spheroplasts, the ability of each cell to concentrate methionine remains the same as in normal bacillary forms, although the volume of the latter is about 1/3 that of the former. Spheroplasts have been shown to contain a greatly reduced phenol-insoluble cell wall fraction as compared to the phenol-soluble fraction and the cytoplasmic protein[11].

In attempting to study the mechanism whereby the cell division cycle regulates increases in cell volume and metabolite concentrating ability, it is important to note that these two parameters are not causatively related; an increase in one does not necessitate an increase in the other. Independent control mechanisms may govern these parameters, because cells preparing to commence division—cells in the late lag phase of growth—increase their ability to concentrate metabolites before they increase in volume. Nevertheless, cells which have begun to divide have synchronized the control over these parameters.

The mechanism whereby bacteria replaceably concentrate metabolites has been extensively studied[7,8]. Evidence for concentration of methionine in *A. fecalis*[13] and of other metabolites in other organisms[7,8], strongly implicates some carrier (pump) in the role of transporting the metabolite into the cell against a concentration gradient. The results obtained in this study suggest that duplication of this carrier takes place synchronously with division or immediately before. This recalls a previous finding suggesting that sites responsible for cell division and for directing cell wall synthesis were duplicated immediately before cell division[10]. These sites were penicillin sensitive. It is tempting to speculate that there may be a relationship between such sites and the metabolite carrier system (pump). Such a relationship is suggested by the lack of increase in methionine concentrating ability in penicillin-induced spheroplasts, which nevertheless retain the ability to synthesize protein and nucleic acids[16]. Similarly, staphylococci treated with penicillin show a striking decrease in their ability to concentrate glutamic acid[18].

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* It would be interesting to correlate these findings with volume determinations from electron microscopic examination of thin sections of bacteria. If volume remains constant, $(\text{width})^2 \times$ cell (length) should be equal for cells from the same culture. This should hold true for cells of different lengths and widths. A rough estimate by the authors using published data for *E. coli*[17] has indicated that this is so.

No information is available as to the structural or chemical nature of the penicillin sensitive sites or of the metabolite carriers. If, indeed, some fraction of the cell wall is involved, it is too small to be observed in terms of a change in uptake of radioactive alanine or methionine.

About 70–80% of the bacterial mass is water. From this it can be concluded that the change in cell volume will reflect a large intake of water. It is tempting to think that the cell operates a water pump in addition to metabolite pumps and that during cell division the number of such pumps is duplicated.

ACKNOWLEDGEMENTS

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THE VISUAL COMPLEX OF THE INSECT: RETINENE IN THE HOUSEFLY

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SUMMARY

Housefly heads were extracted with acetone and the extract chromatographed on columns of alumina. One of the eluted fractions had an absorption maximum at 385 mμ; when reacted with antimony trichloride, a transient blue color, with a maximum absorption at 665 mμ was obtained. This fraction was identified as retinene$_1$. A rhabdomere fraction was isolated from the insect eyes by grinding the heads with buffered sucrose solution and fractionating by centrifugation. The rhabdomere fraction, when acetone extracted, also shows the presence of retinene$_1$. It appears that a visual complex retinene$_1$ isolated from the insect is similar to that of the retinal rods of vertebrates.

INTRODUCTION

The vertebrate photoreceptors are the retinal rods and cones. Each is composed of an inner segment and a rod or cone-shaped outer segment containing the photosensitive pigments, retinene$_1$ or retinene$_2$, (the aldehydes of vitamins A_1 and A_2) which are linked with a protein opsin to form pigment complexes, rhodopsin or porphyropsin for the rods, and iodopsin or cyanopsin for the cones[1]. The photoreceptors of the invertebrates, however, are more varied; they include eyespots, ocelli and compound eyes. The insect eye is a compound one, made up of ommatidia; in the housefly each ommatidium consists of a rhabdome of seven retinula cells. Each retinula cell has a differentiated structure, the rhabdomere, which is the photoreceptor and is similar in structure as well as in function to the vertebrate retinal rods[2-4]. However, little is known of the rhabdomere visual pigments as well as other associated pigments of the eye.

Recently GOLDSMITH[5,6] identified retinene$_1$ in the honeybee, and obtained evidence that the retinene is bound to a protein to form a photosensitive pigment complex with a maximum absorption at 440 mμ. BOWNESS AND WOLKEN[7] also had isolated from the housefly a photosensitive yellow pigment with a maximum absorption at 437 mμ. Since there were similarities in spectroscopic properties between this pigment and some retinene–protein complexes, it was thought that it might be the same type of complex as that found in the honeybee. Since then, we have also

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isolated retinene$_1$ from the heads of houseflies, and it now appears that a visual system in insects is chemically similar to that of the vertebrates.

METHODS AND MATERIALS

Materials

Houseflies (*Musca domestica*) were obtained from the Gulf Research and Development Co.*, and were packed in dry ice during transit. They were kept frozen in the freezer compartment of a refrigerator until they were used. The heads were detached and then separated from the residue by the freezing and sieving techniques of MOOREFIELD[8].

Fractionation and extraction

Three different extraction procedures were used, each of which ended with acetone extraction. Two of the methods follow those of GOLDSMITH[5,6]. Until the acetone extraction was completed, all operations were carried out under red dim light. Approximately 4,000 heads were used for each extraction.

1. The heads were ground with anhydrous sodium sulfate and acetone in a mortar and pestle, until no intact heads could be observed. The mixture was centrifuged at 3,000 rev./min, and the supernatant poured off and retained. The residue was re-extracted with acetone, and, after centrifuging, the supernatant was combined with the first extract. This procedure was continued until the extract appeared to be colorless.

2. The heads were ground with about 80 ml 0.2 *M* phosphate buffer, pH 6.5, and the mixture centrifuged at 12,000 rev./min for 20 min. The residue was extracted with acetone, while the supernatant was fractionated by saturation with ammonium sulfate at 45% and 60% (see ref. 5). The precipitates from the ammonium sulfate treatment were extracted with acetone.

3. The heads were ground with 20 ml of 50% sucrose in *M*/15 phosphate buffer, pH 6.5, and the mixture centrifuged at 3,000 rev./min for 10 min. The residue was extracted with acetone, while the supernatant suspension was diluted with phosphate buffer to a sucrose concentration of 12.5% and centrifuged at 14,000 rev./min for 20 min. The sedimented material was then extracted with acetone.

Chromatography

The initial acetone extract from each of the above preparations (1, 2, 3) was evaporated to dryness, and the residue was dissolved in petroleum ether. The petroleum ether extract was then dried over anhydrous sodium sulfate, evaporated to dryness, redissolved in petroleum ether and chromatographed on alumina (Fisher Adsorption Alumina, 80–200 mesh, obtained from Fisher Scientific Co., Pittsburgh, Pa.). The alumina was "weakened" by exposing it to a water-saturated atmosphere and allowing it to adsorb 5 g water/100 g alumina. The best results were obtained by chromatographing each extract twice. The first column was eluted with 40 ml of 40% acetone in petroleum ether (v/v). The eluate was evaporated to dryness, redissolved in petroleum ether, and applied to a second column. This was developed, and fractions were eluted with some or all of the following solutions: 4%, 10%,

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* Thanks to Dr. A. MALLIS Gulf Research Laboratories for supplying us with houseflies.

17.5 %, 25 % and 40 % acetone in petroleum ether (v/v). The effluent fractions were evaporated to dryness, then stored in a vacuum desiccator in the dark.

Examination of fractions

The dried fractions from the chromatograms were dissolved in 0.6 ml chloroform. The antimony trichloride test was performed by placing 0.25 ml of the chloroform solution in a quartz microcuvette and then adding 0.02 ml acetic anhydride and 0.5 ml antimony trichloride reagent. After mixing, the spectrum of the test solution was read against a blank containing chloroform, acetic anhydride and antimony trichloride, with a Beckman DK-1 recording spectrophotometer. The acetone extracts of the precipitate from the ammonium sulfate fractionation (procedure 2) and the extract of the material which sedimented in 12.5 % sucrose solution (procedure 3) were evaporated to dryness and dissolved in chloroform in order to perform the antimony trichloride test as above.

RESULTS

The result of a typical chromatogram of an acetone extract of the housefly heads is shown in Table I. This table in fact represents an average of several chromatograms; individual chromatograms showed a better separation of one or more of the carotenoids present. Of the various carotenoids obtained, only retinene$_1$ can be identified with certainty. The fraction eluted with 25 % acetone in petroleum ether had an absorption maximum at 385 mμ in chloroform (Fig. 1) and, when reacted with antimony trichloride, a transient blue color with a maximum absorption at 665 mμ was observed (Fig. 2).

TABLE I

FRACTIONS IN ORDER OF ELUTION FROM THE CHROMATOGRAM OF AN ACETONE EXTRACT

Fraction	*Acetone content of eluting fluid (% v/v)*	*Antimony trichloride tests*	
		Maximum absorption mμ	$E_{max} - E_{750}$
1	4	660–690	0.05
2	10	660–690	0.58
3	10	—	—
4	25	625–630	0.80
		690–695	0.27
5	25	665	0.90
6	40	650–660	0.07
7	40	—	—

When the heads were extracted with phosphate buffer, the amount of carotenoid material remaining in the heads (as determined by the intensity of the blue color produced in the antimony trichloride reaction) was slightly less than the amount of carotenoid present in the untreated heads. However, when the heads were treated with the sucrose solution, this decrease was considerably greater. For the material identified as retinene$_1$, the value E_{max}–E_{750} in the antimony trichloride reaction was: 0.90 for the untreated heads, 0.79 when the heads were extracted with phosphate buffer before acetone extraction, and 0.20 when they were treated with sucrose solution before acetone extraction.

Examination of the material removed by the sucrose and phosphate buffer treatments shows a similar result (see Table II). An acetone extract of the material precipitated from the phosphate buffer extract by 45 % saturation with ammonium sulfate showed a maximum absorption peak in the 635–645 mμ range, and an inflection at about 690 mμ when treated with antimony trichloride. Similarly, when the concentration of ammonium sulfate was increased to 60 %, a maximum in the 630–640 mμ range and an inflection at about 690 mμ were found when the precipitate was extracted with acetone and reacted with antimony trichloride. However, when the sucrose extract was diluted with 3 volumes of phosphate buffer, and the resulting precipitate extracted with acetone, the antimony trichloride reaction gave a maximum

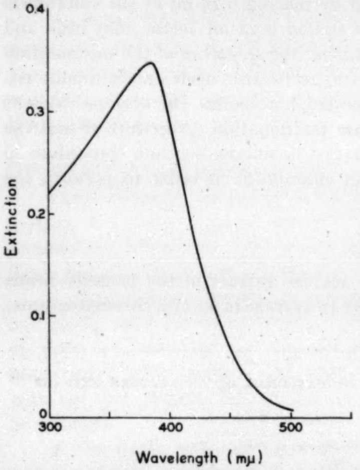

Fig. 1. Absorption spectrum of the retinene-containing fraction in chloroform. (Tracing of Beckman DK-1 spectrophotometer recording.)

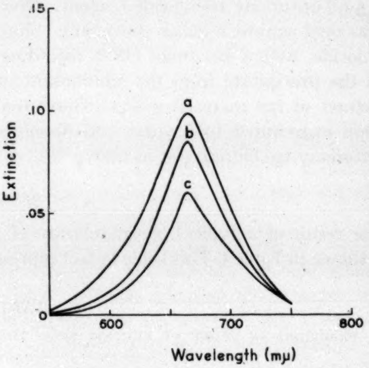

Fig. 2. Absorption spectrum of antimony trichloride reaction of the retinene-containing fraction: a, immediately after mixing; b, approximately 1 min after mixing; c, approximately 2 min after mixing. (Tracing of Beckman DK-1 spectrophotometer recording.)

TABLE II

PHOSPHATE BUFFER AND SUCROSE-IN-PHOSPHATE BUFFER EXTRACTS OF HOUSEFLY HEADS

Material	*Antimony trichloride test of acetone extract*	
	Maximum absorption mμ	$E_{max} - E_{750}$
Procedure 2		
1. Precipitate obtained by 45 % $(NH_4)_2SO_4$ saturation	635–645	0.32
	690	0.20
2. Precipitate obtained by 60 % $(NH_4)_2SO_4$ saturation	630–640	0.11
	690	0.05
Procedure 3		
1. Precipitate obtained on dilution of 50 % sucrose extract	650–660	0.64
	690	0.36

between 650 and 660 mμ—the only case in which the maximum approached the retinene peak of 665 mμ.

The amount of retinene present in the housefly heads may be estimated by comparison with the known $E^{1\%}_{1\,\text{cm}}$ value for retinene in the antimony trichloride reaction[9]. A figure of 0.31 μg of retinene/g fresh weight of heads was obtained, as compared with 0.22 μg of retinene/g fresh weight of honeybee heads reported by GOLDSMITH[5]. Using the average value of 24,500 rhabdomeres per eye[2], this corresponds to $3.7 \cdot 10^7$ molecules of retinene per rhabdomere, a figure within the range of visual pigment molecules per vertebrate retinal rod ($1 \cdot 10^6$–$1 \cdot 10^9$) for the vertebrates so far studied.

DISCUSSION

The presence of retinene$_1$ in the extract indicates that vitamin A should also be present, since, in the visual cycle, retinene$_1$ is reduced to vitamin A$_1$ (see ref. 1). In several chromatograms fractions were obtained whose antimony trichloride tests gave maxima near 620 mμ and 696 mμ, the maxima for vitamins A$_1$ and A$_2$; in some cases these were found in the same fraction (Fig. 3). However, these compounds cannot be positively identified. In all chromatograms where such fractions were obtained, they were eluted from the column before the retinene fraction rather than after it, as would be expected by their structure[10]. Thus, positive identification could not be made. A possible explanation for the absence of vitamin A is that the vitamin may be oxidized to retinene (or changed in some other way) during the procedure. The identification of vitamin A$_2$ is particularly crucial, since it is so far known to occur only in fresh-water fishes and in some amphibians; this would be the first instance of its presence outside of these vertebrate groups.

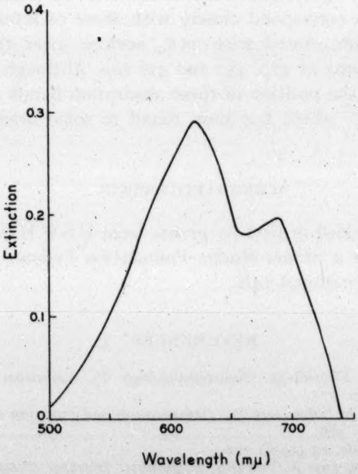

Fig. 3. Absorption spectrum of antimony trichloride reaction of fraction eluted with 17.5% acetone in petroleum ether (v/v) showing maxima at 623 mμ and 693 mμ. (Tracing of Beckman DK-1 spectrophotometer recording.)

GOLDSMITH[5,6] found that 80 % of the retinene-protein material in honeybee heads was extracted with phosphate buffer, and that most of this material could be precipitated by 60 % saturation with ammonium sulfate. The possibility cannot be excluded that a small amount of retinene was extracted with phosphate buffer from the housefly heads, but it was certainly much less than 80 % of the total. Whatever retinene was extracted with phosphate buffer must have been precipitated by 45 % saturation with ammonium sulfate, as there was no trace of the retinene band in the antimony trichloride test of the material precipitated by 60 % saturation (see Table II). Although the phosphate buffer does extract a photosensitive pigment complex, this complex may not be a typical rhodopsin. However, the presence of retinene is indicative of a rhodopsin.

Since the amount of retinene extracted from the housefly heads with phosphate buffer is so small, the light-sensitive yellow pigment previously isolated from the housefly[7] is probably not a rhodopsin. Observations since made on this pigment indicate that it may be related to the xanthommatins described by BUTENANDT, SCHIEDT AND BIEKERT[11], although none of the ommochrome class of pigments has previously been reported to be sensitive to light[12]. Also, there are similarities in spectroscopic properties between this housefly pigment and the photolabile yellow pteridine isolated from *Drosophila* by FORREST AND MITCHELL[13]. However, there is still the possibility that it may contain a retinene–protein complex. Less is known of the protein than of the chromophore, retinene. It has previously been indicated that such proteins would vary in different species[1,14]. A protein soluble in phosphate buffer would indicate a different protein from that of the vertebrate rods.

Of the other fractions eluted from the column, two contained light-stable carotenoids. The fraction eluted with 4 % acetone in petroleum ether was yellow in color and its absorption spectrum in chloroform showed maxima at 486, 455 and 426 mμ. These maxima correspond closely with those of lutein (xanthophyll) (see ref. 15). Another fraction, eluted with 25 % acetone after the retinene fraction, showed absorption maxima at 477, 450 and 427 mμ. Although this material cannot be definitely identified, the position of these absorption bands appears to be closest to "canary-xanthophyll" which has been found in some organisms together with esterified lutein[16].

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Short Communications

On the composition of sheep thyrotropic hormone

Recent communications[1–3] from this laboratory have described the purification and composition of bovine thyrotropic hormone. Starch-gel electrophoresis experiments revealed six biologically active components in purified beef material, two of which were isolated by careful rechromatography on the anion exchanger, diethylaminoethylcellulose. The amino acid compositions of the two isolated thyrotropins were almost identical and a preparation consisting only of active components was shown to contain mannose, fucose, glucosamine and galactosamine. Sheep and whale thyrotropin preparations exhibited chromatographic behavior on diethylaminoethylcellulose similar to that of beef. Several active components were again recognized and approximately the same molecular weight was indicated for the hormones from each species. This paper reports on the composition of hydrolysates of sheep thyrotropin preparations which have been further purified by chromatography on the cation exchanger, carboxymethylcellulose.

The experimental procedures for preparation of the hormone, bioassay, chromatography on the celluloses, starch-gel electrophoresis, and analysis for amino acid and carbohydrate content are as previously described[1–4]. In general, the yields in terms of weight and total units were less than with the beef preparations. Material with a potency of 10–15 U.S.P. units/mg was obtained, a potency similar to that reported by ELLIS[5]. 9 kg of whole sheep pituitaries yielded 2.2 g of 5 unit/mg material which, after chromatography on diethylaminoethylcellulose, yielded two active fractions which emerged from the columns following the bulk of the inactive material (see ref. 3, Fig. 1b). The elution curves from carboxymethylcellulose chromatography and the starch-gel patterns of the sheep material were closely parallel to those reported for beef[2]. The rechromatography on carboxymethylcellulose of the active fraction most strongly retained on diethylaminoethylcellulose yielded the best preparation. From a total of 55 mg (pooled from two runs through the anion exchanger) 21 mg (Sample 1) were obtained from the cation exchanger. A column 1.9 × 17 cm, at pH 6.3 was employed, with a gradient to 0.1 M Na^+ concentration applied over 500 ml after emergence of the first peak (inactive). Starch-gel electrophoresis at pH 9.5 showed Sample 1 to consist of essentially only two components, both of which were demonstrated to be biologically active.

Additional material was obtained by rechromatography, under the same conditions, of the active fraction* first emerging from the anion-exchange columns[3]. The rechromatographed material (10 U.S.P./mg), although consisting chiefly of active components, showed some impurities on starch-gel electrophoresis (Sample 2).

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* It is of interest to note that an impurity was found in this fraction, which was indistinguishable from the active material by starch-gel electrophoresis at pH 9.5. It was recognized by a different mobility in electrophoresis at pH 5.0 and emerged from the carboxymethylcellulose columns before the active material, shortly after application of the gradient to 0.1 M Na^+ concentration.

Because of excessive destruction during hydrolysis prior to amino acid analysis, a third sample was prepared by dissolving a portion of Sample 2 in water and passing it through a column of dextran gel (Sephadex G-25, Pharmacia, Uppsala, Sweden) in order to remove any residual buffer salts[6]. Simultaneously some of the contaminating impurities of Sample 2 were also removed as two protein peaks were found. The second peak to emerge contained the biological activity (Sample 3). Sample 3 showed 2 bands with the expected mobility in starch-gel electrophoresis at pH 9.5. Based on the amount of ammonia present in its hydrolysates, less destruction occurred during hydrolysis of Sample 3.

The carbohydrates of the sheep thyrotropins were found to be the same as for beef, *i.e.*, mannose was the predominating hexose detected by paper chromatography and a weaker but definite spot with the mobility of fucose was also present.

TABLE I

AMINO ACID COMPOSITION OF SHEEP THYROTROPIN PREPARATIONS COMPARED TO BEEF THYROTROPIN *b*

	No. of residues based on a mol. wt. of 28,000				*No. of residues based on a mol. wt. of 28,000**		
	Sheep preparations		*Beef thyrotropin* b		*Sheep preparations*		*Beef thyrotropin* b
	*Found**	*Arbitrarily corrected***	*(ref. 1)*		*Found*	*Arbitrarily corrected***	*(ref. 1)*
Aspartic acid	11.4; 11.2	14.4	14.4	Isoleucine	5.6; 5.4	7.0	6.8
Threonine	12.2; 13.0	16.0	15.6	Leucine	7.2; 7.2	8.5	8.2
Serine	8.3; 8.2	10.5	10.4	Glucosamine	3.4; 1.9	3.3	4.2
Proline	13.2; 13.9	17.2	14.7	Galactosamine	0.8; 0.8	1.0	1.6
Glutamic acid	12.3; 11.1	14.9	14.6	Tyrosine	8.7; 7.6	10.4	10.3
Glycine	8.3; 9.0	11.0	15.3***	Phenylalanine	5.1; 5.1	6.5	6.3
Alanine	10.0; 11.5	13.6	12.3	Ammonia	25.0; 17.8	27.2	21.2
Valine	7.9; 8.8	10.7	11.0	Lysine	10.6; 10.6	13.4	14.8
Half cystine	10.9; 12.6	14.9	16.6	Histidine	3.6; 3.7	4.6	4.8
Methionine	4.1; 3.7	4.9	4.9	Arginine	5.5; 6.1	7.3	7.3

* Values not corrected for moisture or ash. The results were obtained from Sample 1 (left) and Sample 3 (right). A third analysis on another portion of Sample 3 gave similar molar ratios of constituents but recovery could not be calculated because of loss of a small amount of the sample after weighing.

** The results have been arbitrarily corrected to give the same aspartic acid content as in beef thyrotropin in order to directly compare molar ratios of constituents in the two.

*** Free glycine was found in the beef preparations isolated from chromatography in glycine buffers. A value of 11 residues was found for a beef thyrotropin preparation chromatographed in phosphate buffer[2].

Both the anthrone method and an orcinol method[7] indicated the total hexose content of Sample 1 to be 5–8 % and of Sample 2 approx. 5 %. As sufficient material was not available for ash determinations results are not corrected for ash or moisture. In the amino acid analysis two peaks were detected which were ascribed to glucosamine and galactosamine. Material from each peak gave an Elson-Morgan reaction. Table I shows the results of the amino acid analysis together with those for the hexosamines. The values for the latter are low due to destruction during the hydrolysis prior to amino acid analysis[1,2]. The data in Table I show that the ratios of the amino acids found in the sheep thyrotropin are almost identical with those of beef, thus indicating very similar composition for the hormones from the two species. The recovery of residues of amino acids together with the hexose and hexosamine accounts for 70 % of the weight of sample; with beef material recoveries of 80–85 % were obtained (corrected for moisture and ash[1]). Although the low recoveries may be attributed to losses during hydrolysis the possibility of an unrecognized component in the thyrotropins exists. Analysis of other samples of sheep thyrotropins for sialic acids and tryptophan indicates that the two are not constituents of the molecule.

GRÖSCHEL AND LI[8] have recently reported on the carbohydrates of ovine follicle-stimulating hormone and interstitial-cell-stimulating hormone. In comparing the carbohydrate content of ovine thyrotropin with these hormones, a difference in hexoses is found in that the two gonadotropins contain galactose in addition to mannose; galactose is absent in the best preparations of sheep thyrotropin analysed. The 2.44 % sialic acid found in the follicle-stimulating hormone[8] is consistent with the differences in its electrophoretic behavior as compared to thyrotropin[2].

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Purification of chorionic gonadotropin from the urine of patients with trophoblastic tumors

Human chorionic gonadotropin can be detected in the blood and urine of pregnant women and also, in much larger amounts, in the blood and urine of patients* with such trophoblastic tumors as choriocarcinoma, hydatid mole, chorioadenoma destruens, and syncytial endometritis[1]. The gonadotropin titer of these patients can be used as an index of tumor growth or regression. Whereas the hormone from pregnancy urine has been purified and characterized[2], it has not been concentrated or purified thus far from the urine of patients with malignant tumors. We wish to report here

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Abbreviations: Tris, tris(hydroxymethyl)aminomethane; DEAE-, diethylaminoethyl-.
* These patients are referred to collectively in this paper as "tumor patients".

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studies on the purification of the hormone from the latter source, and some properties of the product.

Crude chorionic gonadotropin was obtained from the pooled urine of tumor patients by the kaolin adsorption method of ALBERT[3], and the concentrates were thoroughly dialyzed against distilled water and lyophilized. 1 g of this crude material, which gave an assay value of 150–200 international unite (I.U.)/mg by the mouse-uterine- weight method[4], was dissolved in 40 ml Tris–phosphate buffer, pH 8.6 (0.005 *M* phosphate, 0.04 *M* Tris) and placed on a DEAE-cellulose column (10×4 cm) which had been equilibrated with the same buffer. The applied material was fractionated by stepwise elution, keeping the pH constant while gradually increasing the salt concentration of the eluant with NaCl. The elution schedule and effluent diagram are shown in Fig. 1. Eluate was collected in 7-ml fractions at 3-min time intervals. Protein concentration of the effluent fractions was estimated by measuring their

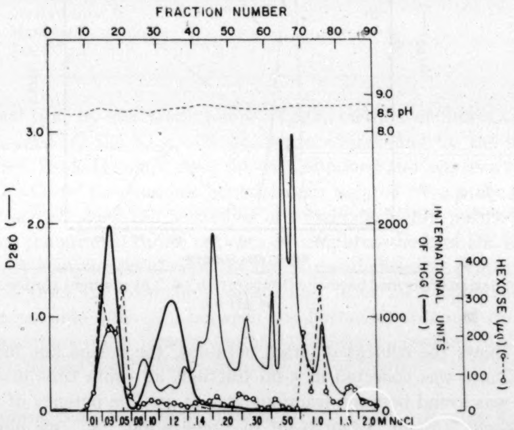

Fig. 1. Fractionation of crude chorionic gonadotropin by stepwise elution on DEAE-cellulose at pH 8.6.

absorption at 280 mμ. Hexose content was determined by the method of SHETLAR[5]. Gonadotropic activity was estimated by the ovarian hyperemia response in immature, female Sprague-Dawley rats[6].

Fig. 1 illustrates that most of the activity can be eluted from the column at a NaCl concentration ranging from 0.01 to 0.12 *M*. The bulk of inert proteins is thus separated from the active material. The effluent containing the hormone was practically colorless, whereas fractions eluted with 0.14 *M* NaCl and up showed a tan to dark-brown color. The appearance of color thus serves almost as a demarcation line. The effluent fractions containing most of the chorionic gonatropin activity were dialyzed and lyophilized. From 1.0 g of crude product (150–200 I.U./mg), we obtained 0.1 g of material with an activity of 1500 I.U./mg. Thus 90% of inactive material was removed while most of the biological activity was retained. We called this preparation "A".

Preparation "A" (0.5 g) was dissolved in 10 ml 0.005 *M* phosphate buffer, pH 6.0, and the solution placed on a DEAE-cellulose column (21 × 2.5 cm). The material was eluted with a straight-line gradient, keeping the pH constant while varying the NaCl concentration. This gradient was obtained by using two chambers of a nine-chamber multiple-gradient device described by PETERSON AND SOBER[7]. The first chamber contained 200 ml 0.005 M phosphate buffer, pH 6.0, 0.08 M NaCl and the second chamber 200 ml of the same buffer containing 0.3 M NaCl.

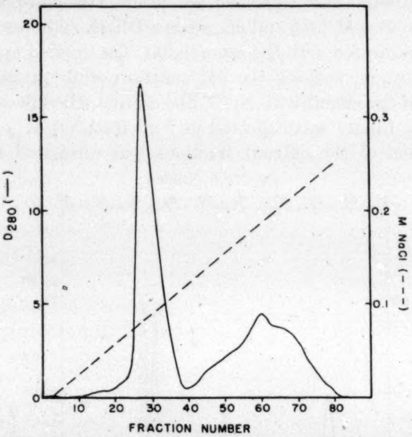

Fig. 2. Fractionation of purified hormone (Preparation "A") by gradient elution on DEAE-cellulose at pH 6.0.

Fig. 2 shows the effluent diagram obtained, the dotted line indicating the salt gradient. Eluate was collected in 5-ml fractions at 3-min time intervals. The bulk of activity was found between fractions 25 and 35. The potency of the product was enhanced about 10-fold. From 0.5 g of preparation "A", we obtained 0.05 g of preparation "A-1". Biological activity data, as determined by the mouse-uterine-weight assay, are summarized in Table I.

We also used a procedure which LEGAULT *et. al.*[8], applied successfully to purify pregnant-mare-serum gonadotropin[8]. The procedure is essentially an adsorption of inactive proteins on $BaCO_3$. The gonadotropin remains in the supernatant, which is then suitably treated to remove barium. Applying this procedure to preparation "A", we were able to increase the biological activity from 1500 I.U./mg to 10–12,000 I.U./mg. However, when this material was examined by moving-boundary electrophoresis it was heterogeneous, exhibiting three distinct components.

TABLE I

BIOLOGICAL ACTIVITY OF CHORIONIC GONADOTROPIN PREPARATIONS

Preparation	*Activity I.U./mg*	*Purification*
Crude	150–200	
"A"	1500	× 10
"A-1"	15000	× 100

Preparation "A-1" was examined by moving-boundary electrophoresis, applying a current of 20 mA and 150 V at pH's 8.5, 6.5, 4.0 and 2.0. After running for 120 min, we observed essentially one symmetrical peak. However, after 180 and 240 min, there was a pronounced skewing effect indicating heterogeneity. The isoelectric point of preparation "A-1", as estimated from electrophoretic mobility data, is between pH 3.8 and 4.0. Hexose content was determined by the cysteine–H_2SO_4 method of DISCHE[9] and by the tryptophane–H_2SO_4 method of SHETLAR[5]. Hexosamine was determined by the ELSON–MORGAN procedure[10] and by the indole–HCl method of DISCHE[11]. Table II summarizes the data obtained and lists as a comparison results obtained by GOT[12] for chorionic gonadotropin isolated from pregnancy urine.

TABLE II

CHARACTERISTICS OF CHORIONIC GONADOTROPIN

	Trophoblastic tumors	*Pregnancy urine*
Activity I.U./mg	15,000	10,–12,000
N %	11.2	10.5
Hexoses %	16.2	11.0
Hexosamine %	6.4	8.7
Hexoses/hexosamine	2.5	1.26
Isoelectric point	3.8–4.0	2.95

The data presented show the feasibility of obtaining highly purified chorionic gonadotropin from the urine of tumor patients by simple methods of DEAE-cellulose chromatography. A comparison of some of the physical-chemical properties of the hormone obtained from the pregnancy source and from the trophoblastic tumor source suggests a possible difference between the hormones obtained from the two sources. This conclusion must remain tentative, however, pending isolation of a preparation of established homogeneity from the tumor sources.

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Preparation of ribonucleic acid in cold concentrated saline and some physico-chemical properties

It has been already reported that the microsomal ribonucleoprotein particles isolated by the method of LITTLEFIELD *et al.*[1] are dissociated to RNA and protein moieties when treated with concentrated saline in the cold, the latter being selectively precipitated under such conditions[2]. This property has been applied to the preparation of RNA[3]. This communication is concerned with the preparation of RNA by this method and some physical chemical properties of the isolated RNA.

A fresh calf liver was cut into strips about 1 cm thickness and frozen in dry ice. For each preparation of RNA, 200 g of the frozen liver was allowed to thaw at 5°, chopped very finely with a meat cleaver and then homogenized in 360 ml 0.25 *M* sucrose solution. The supernatant separated from the homogenate by centrifugation (15 min at 14,000 × *g* in the cold) was the starting material for the preparation.

To this supernatant, solid NaCl was added to a final concentration of 2 *M* under constant stirring, and kept overnight in the cold. After centrifugation for 15 min at 4,000 × *g*, the supernatant was carefully removed. The loosely packed sediment was collected and washed several times with 2 *M* NaCl in the cold. The gelatinous pellet obtained was suspended in 0.02 *M* KH_2PO_4–K_2HPO_4 buffer (pH 7.0) with the aid of a glass homogenizer and then the solution was centrifuged for 15 min at 14,000 × *g*. If the dissolution was incomplete, such a procedure was further repeated. The clear supernatant thus obtained was further submitted twice to the treatment with strong saline and dissolution in the 0.02 *M* phosphate buffer as described above. The pellet obtained after such purification procedures was suspended in the 0.02 *M* phosphate buffer and dialysed against the same buffer overnight in the cold. During dialysis, the solution became clear, the small amount of insoluble material being removed by centrifugation for 30 min at 20,000 × *g*.

About half of the RNA in the original mitochondrial supernatant was recovered in the final RNA solution, in which none or only a trace of DNA was detected by the diphenylamine test, and 1–3 % protein contaminant was found by LOWRY's method[4]. The maximum and minimum of its absorbancy was found at 258 and 230 mμ respectively, average $A_{230\,\mathrm{m}\mu}/A_{258\,\mathrm{m}\mu}$ being 0.475.

The RNA prepared by this procedure is electrophoretically homogeneous, its ascending and descending mobilities in phosphate–saline buffer (pH 7.0, I 0.1) at 0° being -14.5 and $-13.6 \cdot 10^{-5}$ $\mathrm{cm}^2 \cdot \mathrm{V}^{-1} \cdot \mathrm{sec}^{-1}$ respectively. Ultracentrifugation showed three components, the main one being the slowest (Fig. 1a). The sedimentation coefficient ($S^{\circ}_{20,\,w}$), were 11 S, 16 S and 20 S respectively. Viscosity measurements were carried out at 25° in an Ostwald viscosimeter having flow time of approximately 120 sec for water. The intrinsic viscosity $[\eta]$ of the RNA in 0.02 *M* potassium phosphate buffer (pH 7.0) was found to be 0.30 by extrapolation to zero concentration, which was in good agreement with the value calculated from HUGGINS' equation[5]. Assuming that the various components are homologous with regard to frictional properties, the intrinsic viscosity of 0.30 for the mixture was applied to SCHERAGA-MANDELKERN equation[6] to estimate the sedimentation-viscosity molecular weight of the 11 S component. The value calculated was $28 \cdot 10^4$.

Abbreviation: RNA, ribonucleic acid.

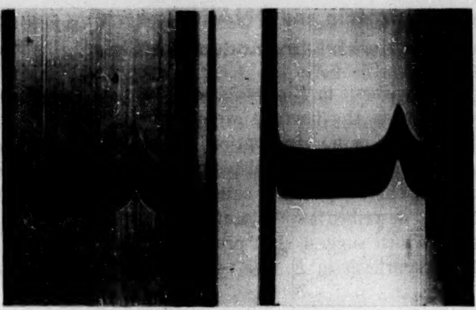

Fig. 1. Sedimentation photographs of (a) the RNA prepared by cold 2 M NaCl and (b) the heat-treated RNA (85°, 15 min). (a) Taken after run for 45 min at 47,300 rev./min. (b) Taken after run for 30 min at 60,000 rev./min. Concentration, 0.67 %; solvent, 0.02 M KH_2PO_4–K_2HPO_4 buffer (pH 7.0); bar angle 35° and 40°, respectively.

The absorbancy at 258 mμ in 0.02 M potassium phosphate buffer (pH 7.0) of the RNA rose by 18 % when the temperature was raised from 10° to 85° and more than 90 % recovery in the absorbancy was observed after cooling it again to 10°. The sedimentation patterns were, however, irreversibly altered; only a slower but remarkably homogeneous peak (8 S) was obtained (Fig. 1b). Its calculated molecular weight was about 10^5, suggesting that this component probably corresponds to the sub-unit RNA described by HALL AND DOTY[7].

As the molecular weight of the RNA prepared by aqueous phenol has been reported to be in the order of a million[8–11], the RNA isolated by the present method seems to have suffered considerable degradation during the isolation procedures.

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Synthesis of protein and DNA in *Escherichia coli* treated with 5-bromodeoxyuridine

Resumption of DNA synthesis in *Escherichia coli* has been reported to require prior synthesis of protein when the bacteria were pretreated with a sulfur mustard[1], bis(β-dichloroethyl)sulfide, or u.v.-irradiation[2]. It has also been demonstrated that when u.v.-treated *E. coli* cells are resuspended in growth medium, inhibition of protein synthesis with chloramphenicol caused a decrease in the number of viable cells[3]. This effect was described as "chloramphenicol death". This communication reports experiments which suggest that protein synthesis is not a prerequisite for resumption of DNA synthesis in *E. coli* 15T$^-$ after a prior exposure to 5-bromodeoxyuridine.

E. coli 15T$^-$ was grown on a synthetic medium[4] containing 1 μg/ml thymine and 100 μg/ml BrUDR. The cells were grown with aeration for 2 h at 37°. At the end of the growth period, the viable count (measured on nutrient-agar plates after 18 h) was reduced to less than 3 % of the inoculum. The cells were harvested, washed with mineral medium, resuspended in three flasks and aerated at 37°. The time at which the cells were resuspended in fresh media corresponds to "0 min" in the figures. Flask 1 contained synthetic medium plus 1 μg/ml of thymine. Flasks 2 and 3 contained synthetic medium, 1 μg/ml thymine and 75 μg/ml chloramphenicol added at 0 min (flask 2) and after 15 min (flask 3). At intervals of 30 min, samples were withdrawn for viability, DNA (diphenylamine[5]) and RNA (orcinol[6]) determinations. Growth was followed turbidimetrically at 420 mμ in a Klett-Summerson photoelectric colorimeter. Cell count was determined with a Petroff-Hauser counter.

As shown in Fig. 1, no net protein synthesis (based on turbidity measurements[7]) occurred in the resuspended cells, and there was no change in cell count over a 120-min period (Fig. 1). RNA content of the cells increased by a factor of about 1.4 in the presence or absence of chloramphenicol (Fig. 2). DNA increased by a factor of 3.6 in the absence of chloramphenicol, and 2.8 in the presence of chloramphenicol (Fig. 3). This increase in DNA content was unrelated to either cell number (Fig. 1) or viability (Fig. 4). Although the pattern of cell death was variable at the three conditions (Fig. 4), in each instance the number of surviving cells at the end of 2 h was lower than at "0 time". The temporary restoration of viability produced by chloramphenicol added at "0 min" is similar to the "restoration and second death" of the same strain subjected to u.v.-radiation as described by BARNER AND COHEN[8].

These results demonstrating continued synthesis of DNA while cell count remains constant are similar to those reported by OKAZAKI AND OKAZAKI[9]. They showed that addition of thymidine to a deoxyribose-requiring strain of *Lactobacillus acidophillus*, previously deprived of a deoxyribose source, resulted in active DNA synthesis with comparatively little increase in protein, RNA or cell number. Their results are also similar in that chloramphenicol had little effect on DNA synthesis. From similar studies, others have concluded that u.v.[2]- or mustard[1]-treated *E. coli* require protein synthesis prior to initiation of DNA synthesis.

These differences in the requirement of protein synthesis for initiation of DNA duplication may be due to differences in cell-protein damage produced by mustards,

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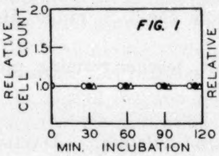
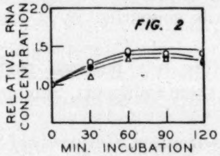
Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; BrUDR, 5-bromodeoxyuridine.

u.v.-light, deoxyribose limitation and BrUDR. It has been established that BrUDR inhibits DNA synthesis by limiting utilization of thymidylate[10], or it may be incorporated in place of thymidine[11] into DNA. No other effects of this compound are known. Similarly, limitation of deoxyribose would be expected to interfere primarily with DNA synthesis. Alkylating agents and u.v.-irradiation are known to produce changes in both DNA and protein structure[12]. Thus it is reasonable to expect that BrUDR or deoxyribose limitation produce a relatively specific effect on cellular DNA





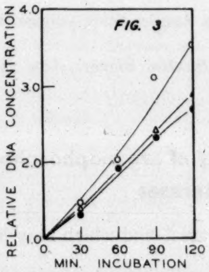
o — O CLMP; ● — CLMP at "o min"; △ — CLMP at "15 min".

Fig. 1. Effect of chloramphenicol (clmp) on cell count and turbidity of *E. coli* 15T⁻ after BrUDR treatment. Cells were grown for 2 h in the presence of 100 µg/ml BrUDR and then resuspended in synthetic medium plus 1 µg/ml thymine at "o min". 75 µg/ml chloramphenicol was added at times indicated.

$$\text{Relative turbidity or cell count} = \frac{\text{turbidity or cell count at } t \text{ min after resuspension}}{\text{turbidity or cell count at the time of resuspension}}$$

Fig. 2. Effect of chloramphenicol on RNA in *E. coli* 15T⁻ after BrUDR treatment. Cells were grown in the presence of 100 µg/ml BrUDR for 2 h and then resuspended in synthetic medium plus 1 µg/ml thymine at "o min". 75 µg/ml chloramphenicol was added at times indicated.

$$\text{Relative concentration of RNA} = \frac{\text{concentration at } t \text{ min after resuspension}}{\text{concentration at the time of resuspension}}$$



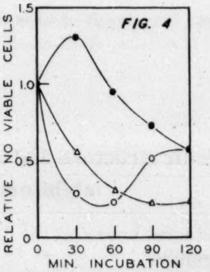


Fig. 3. Effect of chloramphenicol on DNA synthesis in *E. coli* 15T⁻ after BrUDR treatment. Cells were grown in the presence of 100 µg/ml BrUDR for 2 h, and then resuspended in synthetic medium plus 1 µg/ml thymine at "o min". 75 µg/ml chloramphenicol was added at times indicated

$$\text{Relative concentration of DNA} = \frac{\text{concentration at } t \text{ min after resuspension}}{\text{concentration at the time of resuspension}}$$

Fig. 4. Effect of chloramphenicol on viability of *E. coli* 15T⁻ after BrUDR treatment. Cells were grown in the presence of 100 µg/ml BrUDR for 2 h, and then resuspended in synthetic medium plus 1 µg/ml thymine at "o min". 75 µg/ml chloramphenicol was added at times indicated.

$$\text{Relative number of viable cells} = \frac{\text{viable cells at } t \text{ min after resuspension}}{\text{viable cells at the time of resuspension}}$$

whereas u.v.-light or mustards cause additional damage to functional proteins to the extent that renewal is necessary prior to continued DNA synthesis.

Although BrUDR is known to produce a primary effect on DNA, there is a continued synthesis of DNA in BrUDR-treated *E. coli* which serves no apparent purpose for survival or cell division. This useless DNA synthesis without concomitant cell division suggests that the inability of *E. coli* to survive in growth medium after exposure to high concentrations of BrUDR is due primarily to loss of DNA function rather than failure to produce DNA.

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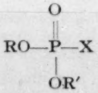
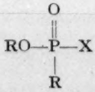
Electronic structure and activity of organophosphorus inhibitors of esterases

The group of substances known as the "nerve gases" and which may be represented by the general formulas



$$RO-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OR'}{|}}{P}}-X$$

or



$$RO-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle R}{|}}{P}}-X$$

where X is –F, –CN or $-O.C_6H_4 \cdot NO_2$ and R and R′ are alkyl groups (and where the O's may sometimes be replaced by N or S) are highly specific inhibitors of enzymes which possess esterase activity, particularly cholinesterase and acetylcholinesterase[1]. Their mode of action bears striking similarity to the first stage of the process of enzymic hydrolysis of the biochemical substrates themselves: the substituted phosphoryl group of the inhibitors is transferred to the enzyme with the resultant formation of a phosphorylated enzyme (analogous to the acylated enzymes formed in the reactions with biochemical substrates). The inhibition of the enzyme is due to the stability of its phosphorylated derivative, the active center being thus practically permanently blocked (for possible reasons of this stability, see ref. 2).

The similarity of behaviour of the organophosphorus inhibitors and the biochemical substrates in the first stage of their interaction with the esterases suggests the existence of important structural analogies between these two types of compounds.

Now, it has been shown by PULLMAN AND PULLMAN[3] that practically all the fundamental biochemical substrates undergoing enzymic hydrolysis have the common feature of undergoing this reaction on a π-electron-deficient bond. In related series of substrates the ease of enzymic hydrolysis seems to be the greater the greater the electron deficiency of the susceptible bond.

Calculations carried out for representative organophosphorus inhibitors show that the hydrolysable bond of these compounds has the same π-electron-deficient character as the analogous bonds of the natural substrates (Fig. 1). This situation accounts largely for the observed similarity in the first stage of the interaction of these two types of compounds with the enzymes.

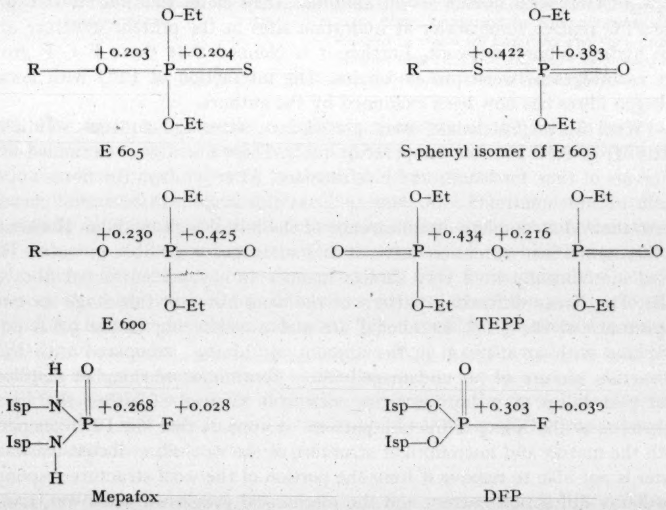


Fig. 1. Formal positive charges on the hydrolysable bond of the organophosphorus inhibitors of esterases (Et = ethyl, Isp = isopropyl, R = *p*-nitrophenyl).

Moreover, it may be remarked that the inhibitory power of related organophosphorus derivatives seems to parallel the value of the positive charge on the P atom. Thus, following ALDRIDGE[4,5] the inhibitory power increases in the series E 605, S-phenyl isomer of E 605, E 600 or when passing from mepafox to DFP. This result may probably be considered as indicating that when other conditions, especially the steric ones, are similar the inhibitory power depends essentially on the affinity of the P atom for an electron-rich center of the enzyme.

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Interaction between perfluoro-octanoic acid and the fibrous proteins keratin and collagen

KLEVENS and others[1-3] have examined the interaction of perfluoro-octanoic acid ($C_7F_{15}COOH$) with bovine serum albumin. They claim that the F–C–F groups in the PFO replace some water at hydration sites in the protein structure and enter the hydrogen-bond network. Further it is claimed that these F–C–F groups can act as bridges between protein chains. The interaction of PFO with keratin and collagen fibres has now been examined by the authors.

Wool fibres (Corriedale) were placed in a saturated aqueous solution (about 0.023 *M*) of PFO at room temperature (20°). These fibres were examined at regular intervals of time for length and birefringence. After 30 days the fibres appeared to reach a stable contraction of about 4%. At this stage load-extension curves of the fibres showed a complete disappearance of the initial high-modulus Hookean region of the normal load-extension curve of an undamaged wool fibre in water. The fibres acted mechanically in a very similar manner to a supercontracted fibre in conc. LiBr. The X-ray diffraction pattern of the wool fibres at this stage showed a disappearance of the 5.1 Å meridional arc and a weakening of the 9.8 Å equatorial reflection with an increase in the amount of "arcing" compared with the X-ray diffraction picture of an undamaged fibre. Continuous washing in distilled water over periods up to 7 days gave no noticeable reversal of either the mechanical properties or the X-ray diffraction pattern. It appears that the PFO has penetrated both the matrix and microfibrillar structure of the wool fibre. Extensive washing in water is not able to remove it from the portion of the wool structure responsible for the X-ray diffraction pattern and the mechanical properties when wet, namely the microfibrils[4]. The PFO has disorganized the α-helices probably by taking part in the inter- and intra-chain hydrogen bonding. The inter-helical distance appears to be preserved although there is some disorganization indicated by the change in the 9.8 Å equatorial reflection.

Wool fibres placed in a PFO solution in D_2O at room temperature for 30 days and subsequently washed in D_2O showed a complete hydrogen to deuterium exchange of the hydrogen in the amide –NH (detection technique described by FRASER AND

Abbreviation: PFO, perfluoro-octanoic acid.

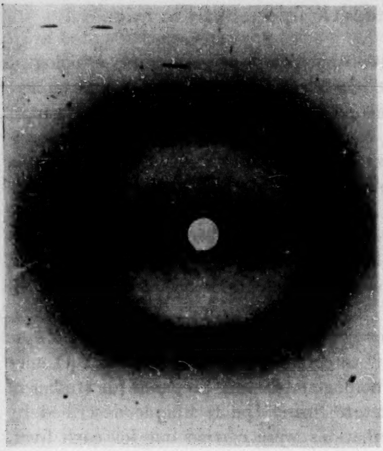

Fig. 1. X-ray diffraction pattern of normal wool keratin fibres.

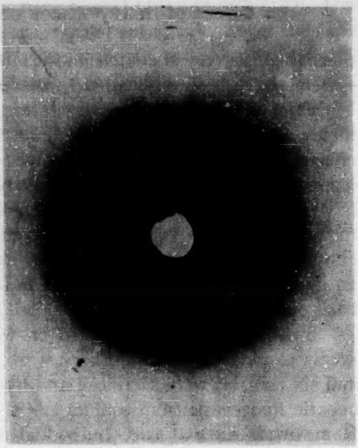

Fig. 2. X-ray diffraction pattern of wool keratin fibres immersed in PFO for 10 weeks, washed in water for two days and then dried. Similar conditions of exposure were used for both patterns of Figs. 1 and 2.

MacRae[5]). This again indicates the complete penetration of the keratin structure by the PFO since, with normal wool fibres in D_2O at room temperature, the hydrogen to deuterium exchange proceeds only to about 70 % completion.

Collagen fibres from rat tail when placed in a satd. aq. PFO at room temperature contract rapidly lengthwise and swell diametrically. After about 10 min the rate of change of diameter and length has decreased considerably. X-ray diffraction photographs of collagen fibres that have been treated with PFO for 20 min show a major weakening in the high-angle X-ray diffraction pattern when compared with the pattern obtained from the same fibre untreated. When the fibre was allowed to remain in the PFO for an extended period (more than two days) it contracted into a rubber-like material, which was found difficult to handle.

The PFO apparently is capable of rapidly entering into the crystalline structure of the collagen fibre, as it does in keratin, by its ability to enter the hydrogen-bonded network which stabilises the crystalline phase.

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Effect of heavy water on hepatic lipogenesis in mice

Since the discovery of deuterium the effects of this isotope on many diverse biological systems have been studied[1]. In general it has been shown that deuterium in high concentrations is toxic to all organisms, the degree of toxicity being greater in more highly organized organisms. BARBOUR[2–4] carried out a number of studies on the effects of D_2O ingestion on mice and concluded that when the body water is only 0.2 saturated with D_2O the mice survive but show elevated metabolism. KATZ *et al.*[5] have demonstrated that mice can be maintained on 25 % D_2O in their drinking water for periods up to a year with no adverse effects. Our own observations that tissue-culture cells maintained in 25 % D_2O exhibit lipid-containing inclusions[6,7] prompted an investigation into the effects of D_2O feeding on hepatic lipogenesis, which is the basis of this report.

Swiss mice were maintained on water containing 25 % D_2O, with *ad libitum* access to food and water. Every week, for three consecutive weeks, six D_2O mice and six controls were sacrificed and liver homogenates were prepared for studies of hepatic lipogenesis from sodium [2-^{14}C]acetate, using the technique described by RABINOWITZ AND GURIN[8]. Individual incubations were carried out for each liver. Cholesterol and fatty acids were isolated by previously described methods[9]. The results are presented in Table I.

TABLE I

EFFECT OF D_2O FEEDING (25 %) ON HEPATIC FAT SYNTHESIS IN MICE

Weeks	*Diet*	*Cholesterolgenesis*		*Fatty acid synthesis*	
		Counts/min/mg C × 10³	*% Reduction*	*Counts/min/mg C × 10³*	*% Reduction*
	H_2O	1.57 ± 0.16*		9.50 ± 0.99	
1	D_2O	1.46 ± 0.18	8	4.92 ± 0.69	49
				(0.01 < P < 0.001)	
	H_2O	1.58 ± 0.14		3.26 ± 0.54	
2	D_2O	1.12 ± 0.16	30	3.31 ± 0.17	—
		(P = 0.05)			
	H_2O	1.10 ± 0.21		5.55 ± 0.17	
3	D_2O	0.69 ± 0.10	38	3.15 ± 0.22	44
		(P = 0.10)		(P < 0.001)	

* Standard error of the mean.

It is evident that in D_2O-fed mice there is a general reduction in hepatic lipogenesis which becomes more pronounced with continuing D_2O ingestion. Histological examination of livers from D_2O-fed mice (carried out by Dr. V. DEFENDI) showed an increase in sudanophilic material over the controls and a progressive increase in sudanophilia as D_2O imbibition continues. Accumulation of liver cholesterol has been shown to inhibit cholesterogenesis[10,11] and our results might be explained on this basis.

To investigate the effect of D_2O concentration in the liver homogenate parallel experiments were carried out in which the buffer solution was taken to dryness under reduced pressure and then reconstituted in water or in D_2O ($> 99.5\,\%$ D_2O). The pH of both solutions was 7. In the homogenates prepared with heavy water the D_2O concentration was about 75 %. In each experiment six incubations were carried out with each buffer solution. The results are summarized in Table II.

In homogenates containing D_2O there was a considerable enhancement of lipogenesis. We may assume that the lipid accumulation in the livers of the D_2O-fed mice was due to accelerated lipogenesis during the early days of feeding. The influence of D_2O on various enzyme systems was studied by a number of early investigators[1] with effects ranging from enhancement of activity to inhibition. The mechanisms underlying our observations await further study.

TABLE II

EFFECT OF D_2O INCUBATION ON HEPATIC FAT SYNTHESIS IN MICE

Expt.	*Medium*	*Cholesterolgenesis*		*Fatty acid synthesis*	
		Counts/min/mg C × 10^4	*% increase*	*Counts/min/mg C × 10^4*	*% increase*
	H_2O	1.00 ± 0.43*		2.84 ± 0.09	
1	D_2O	1.31 ± 0.05	31	3.24 ± 0.05	14
		($P < 0.001$)		($0.01 > P > 0.001$)	
	H_2O	1.29 ± 0.04		2.55 ± 0.14	
2	D_2O	2.12 ± 0.10	65	3.69 ± 0.09	45
		($P < 0.001$)		($P < 0.001$)	

* Standard error of the mean.

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Octanoate oxidation in the cerium-induced fatty liver

Cerium and certain other rare earths, when administered intravenously to rats, produce a fatty liver[1,2]. The purpose of the experiments reported here was to determine the relationship, if any, between fatty acid oxidation and the lipid and the cerium content of the liver after the administration of rare-earth metal.

Female Carworth-Farms-Nelson strain rats weighing between 150 and 200 g were maintained on a Dietrich and Gambrill rat laboratory chow. Fatty livers were produced by administering cerium (2 mg/kg) as the chloride by a tail-vein injection[1,2]. The rats were killed at various times after the rare-earth injection. All animals were fasted 24 h before sacrifice. The oxidation of octanoic acid by rat-liver mitochondria[3] was measured using the procedure described by LEHNINGER[4]. Redistilled octanoic acid was prepared as the potassium salt for use in all Warburg experiments. The results for the octanoate oxidation were expressed as µmoles O_2/mgN/h. A semimicro-Kjeldahl method[5] was used for analysis of the tissue nitrogen. In another group of rats the amount of cerium remaining in the liver after the injection was determined from the distribution of tracer ^{144}Ce(25–30 µC) that had been incorporated into the 2 mg/kg cerium dose. The livers were wet ashed with boiling HNO_3 and an aliquot of this solution was used to assay the radioactivity. The measurements of radioactivity were made with a NaI crystal and a Nuclear-Chicago medical spectrometer (model 132).

Fig. 1 shows a practically complete inhibition of octanoate oxidation by liver mitochondria obtained from rats killed 2 days after the administration of stable cerium. The wide range of values observed at 1 and 6 days is similar to the variation of liver lipids during the onset and recovery phases of fatty infiltration, respectively, in rats exposed to cerium[2]. The curve shown in Fig. 2 indicates that the cerium rapidly localizes in the liver (about 70 % of the dose injected) and it remains in the liver at the same high level, even though the liver fat returns to normal after a week[2]. The evidence shown here would imply that the development of the fatty liver and the recovery of the liver from the lipid infiltration depend on the ability of the mitochondrial fatty acid oxidizing systems to function properly. Furthermore, lipid mobilization from extrahepatic tissues appears to be a significant source of the abnormal accumulation of liver lipids after a single cerium injection[6].

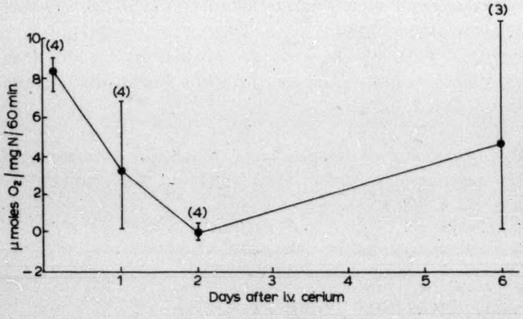

Fig. 1. Fatty acid oxidation in cerium (2 mg/kg)-treated rats. All animals fasted 24 h before being killed. The number of rats used for each determination is shown in the parenthesis. The mean value is represented by the solid circles and the range is indicated by the vertical bar which passes through each mean value.

The large metal concentration in the liver and the changes seen in the oxidase system do not at first glance seem to have any relationship; however, the action of cerium on the mitochondrial system studied could be modified if the chemical form of cerium in the liver were altered with time. At present we have no knowledge of what chemical form the cerium might have in the liver. It is well known that the rare earths form complexes with a wide variety of biological substances *in vitro*[10–12], but a better knowledge of the chemical nature of cerium *in vivo* is needed, especially under the conditions that produce fatty-liver degeneration. The work of BAMANN *et al.*[7–9] has emphasized the important "phosphatase-like" activity of many of the rare-earth metal hydroxides, including that of cerium. Whether dephosphorylation of mitochondrial nucleotides by cerium *in vivo* can explain the oxidative change reported here is a question that deserves attention.

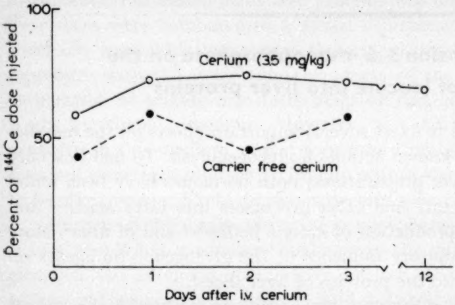

Fig. 2. Localization of cerium in rat liver. Each mean value represents data from 4 rats. The carrier-free cerium is the lowest carrier level of ^{144}Ce available from Oak Ridge National Laboratory (approx. 0.02 mg cerium/kg under the condition of these experiments).

One of us (F.B.) is a summer participant from Idaho State College. This investigation was carried out under contract with the United States Atomic Energy Commission.

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Preliminary Notes

The action of adenosine 3′,5′-monophosphate on the incorporation of leucine into liver proteins

Glucagon and adrenalin are known to exert several important effects on the metabolism of the liver besides their well-known action on glycogenolysis. To mention only the effects observed on isolated liver preparations, both hormones have been found to inhibit the incorporation of acetate and other precursors into fatty acids[1,2] and cholesterol[3], and to stimulate the production of ketone bodies[2,4] and of urea[5]. More recently, we have reported an inhibitory influence of the glycogenolytic agents on the incorporation of amino acids into the proteins of liver slices[6].

According to the investigations of SUTHERLAND and his co-workers[7,8], the earliest known metabolic event involved in the glycogenolytic effect of glucagon or adrenalin is the formation from ATP of the cyclic nucleotide adenosine 3′,5′-monophosphate. This nucleotide then promotes the conversion of inactive dephospho-phosphorylase into the phosphorylated active form of the enzyme, which in turn is responsible for the increased rate of phosphorolysis of glycogen.

It has been shown[9] that 3′,5′-AMP, when added in small amounts to liver slices incubated in a phosphate–saline medium, stimulates glucose production and glycogen breakdown. Earlier experiments[4] from this laboratory have demonstrated that the nucleotide also exerts a ketogenic effect under the same conditions. The results re-

TABLE I

ACTION OF GLUCAGON AND 3′,5′-AMP ON LEUCINE INCORPORATION

About 170 mg of rabbit-liver slices were incubated in 3 ml 0.12 M NaCl, 0.02 M potassium phosphate, pH 7.4, and 10^{-5} M L-leucine uniformly labelled with ^{14}C and corresponding to a radioactivity of 0.05 μC/ml. When added, glucagon was present at a concentration of 33 μg/ml and the nucleotides were $3.3 \cdot 10^{-4}$ M. The flasks were incubated with shaking for 45 min at 37° under pure O_2. The proteins were isolated by a conventional procedure involving precipitation and extensive washing with trichloroacetic acid, removal of nucleic acids and lipids, and treatment with performic acid.

Expt.	*μC × 10^6 incorporated/mg protein*		
	Control	*Glucagon*	*3′,5′-AMP*
78	122	82 (— 33 %)	77 (— 37 %)
79	370	270 (— 27 %)	230 (— 38 %)
80	208	178 (— 15 %)	155 (— 25 %)
81	89	74 (— 17 %)	70 (— 21 %)
86	100	86 (— 14 %)	82 (— 18 %)
3′-AMP	101 (+ 1 %)		
87	166		119 (— 28 %)
3′-AMP	167 (+ 1 %)		
5′-AMP	160 (— 4 %)		

Abbreviations: ATP, adenosine triphosphate; 3′,5′-AMP, adenosine 3′,5′-monophosphate; 3′-AMP, adenosine 3′-monophosphate; 5′-AMP, adenosine 5′-monophosphate.

ported here and summarized in Table I indicate that 3′,5′-AMP likewise inhibits the incorporation of amino acids into the proteins of rat-liver slices incubated in a phosphate–saline medium, to at least the same extent as does glucagon.

In other experiments not described here, little or no effect of 3′,5′-AMP on the incorporation of amino acids into proteins was observed when either rat- or rabbit-liver slices were incubated in a Krebs–bicarbonate medium, whereas glucagon and adrenalin were inhibitory under these conditions[6]. Neither has it been possible to reproduce with the nucleotide the effects of the glycogenolytic agents on the incorporation of acetate into fatty acids or cholesterol in liver slices incubated in a Krebs–bicarbonate medium. However, 3′,5′-AMP also does not stimulate glucogenolysis in this medium, and it seems very probable, therefore, that the nucleotide does not penetrate sufficiently rapidly within the cells or is too rapidly inactivated to be effective in a Krebs–bicarbonate medium.

In view of the results obtained in phosphate–saline, it is reasonable to conclude that 3′,5′-AMP is a common mediator in the various metabolic effects exerted by glucagon and adrenalin on the liver. On the other hand, it seems unlikely that the actions of the cyclic nucleotide on lipid metabolism could be secondary consequences of the activation of phosphorylase[3]. Various experiments have led to the same conclusion with respect to the effect of the glycogenolytic agents on protein synthesis. It appears, therefore, that 3′,5′-AMP must influence one or more additional enzyme systems besides that involved in the activation of phosphorylase. The nature of these systems raises interesting problems.

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The nature of the fluorescence of an enzyme-reduced coenzyme-reduced substrate complex

This paper presents evidence that L-glutamate decreases the dissociation of the glutamic dehydrogenase–TPNH complex, and that the increase in the intensity of fluorescence of that complex caused by the addition of L-glutamate is due solely to the increase in the amount of TPNH bound rigidly to the enzyme surface.

Abbreviations: TPNH, DPNH, reduced tri- and diphosphopyridine nucleotides; GAD, glutamic dehydrogenase.

We have shown previously that the intensity of fluorescence of TPNH (or DPNH) is increased whenever the molecule is bound on any surface which decreases the flexibility of the reduced coenzyme molecule, and have suggested that such increases in fluorescence intensity of reduced coenzymes (referred to hereafter as Δf), which occur upon addition of their apoenzymes, are explained by such binding[1]. We have also shown that the Δf caused by the addition of GAD is a direct measure of the GAD–TPNH complex[2]. From the fact that α-ketoglutarate is not bound to the enzyme in the absence of the reduced nicotinamide ring, from the obligatory order of addition of reactants, and from stereochemical considerations, it was concluded that the α-carboxyl group of α-ketoglutarate is bound to the face of the reduced nicotinamide ring of TPNH. WINER AND SCHWERT[3] had already shown that the Δf of TPNH, caused by the addition of GAD, is further increased by glutamate, which may be presumed to form a ternary complex with its α-carboxyl group bonded to the reduced nicotinamide ring in a manner similar to that of α-ketoglutarate.

The above considerations predicted the possibility that glutamate so bonded could suppress the dissociation of the GAD–TPNH complex. That this predicted suppression actually occurs is shown in Fig. 1. The negative reciprocal of the apparent dissociation constant is, of course, the intercept of the curve on the abscissa. It is apparent that, at least within this range of experimental conditions, increasing the concentration of L-glutamate progressively lowers the apparent dissociation constant of TPNH.

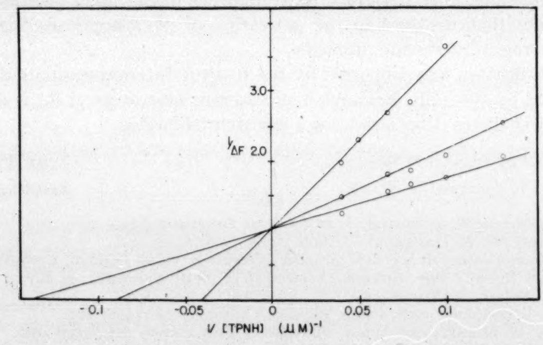

Fig. 1. Lineweaver–Burk plots of complex formation of TPNH at different concentrations of L-glutamate. Each cuvette contained 0.8 mg/ml GAD, TPNH as indicated in 0.16 M phosphate buffer, pH 7.7. L-glutamate concentrations were: (A) 0; (B) 0.3 mM; (C) 0.85 mM. The exciting wavelength was 370 mμ. Δf was measured as the fluorescence intensity at the wavelength of maximum fluorescence corrected for the separately measured fluorescence intensities of TPNH and GAD at the same wavelength.

Although such suppression could be considered intuitively as simply a lowering of the rate of the dissociation of TPNH from the enzyme, the formal kinetics of the proposed mechanism provide us with additional information. Consider the following system:

$$T + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C_1 \qquad (1) \qquad\qquad C_1 + G \underset{k_4}{\overset{k_3}{\rightleftharpoons}} C_2 \qquad (2)$$

where T = TPNH, E = free enzyme, C_1 = enzyme–TPNH complex, G = L-glutamate, and C_2 = enzyme–TPNH–glutamate complex. If we now make the critical assumption that the molar coefficient of fluorescence is identical for C_1 and C_2, we obtain the following expression for $\Delta f = ([C_1] + [C_2])$ in arbitrary units:

$$\frac{1}{\Delta f} = \frac{1}{[E_t]} + \frac{K_T}{[T]\,[E_t]}\left(\frac{1}{1 + \frac{[G]}{K_G}}\right) \tag{3}$$

where $K_T = k_2/k_1$; $K_G = k_4/k_3$; E_t = total enzyme. In the absence of glutamate, the equation reduces to:

$$\frac{1}{\Delta f} = \frac{1}{[E_t]} + \frac{K_T}{[T]\,[E_t]} \tag{4}$$

Equations (3) and (4) represent straight lines with identical intercepts on the ordinate $(1/[E_t])$ but different intercepts on the abscissa. The data shown in Fig. 1 are therefore consistent with this model, and the assumption that the molar fluorescence intensity of the binary complex and of the ternary complex are identical is not only justified, but required.

Where $1/\Delta f = 0$, eqn. (3) simplifies to: $$K_G = \frac{-[T]\,[G]}{K_T + [T]} \tag{5}$$

Using the value of K_A from curve A (eqn. 4), and the value of (T) obtained from the intercepts on the abscissa of curves B and C at their respective values of (G), we can calculate values for K_G. This calculation gives values for K_G of $2.6 \cdot 10^{-4}$ and $3.6 \cdot 10^{-4}$ M respectively. The agreement of these values, within the experimental error of their determination, provides a totally independent check on the agreement of the data with the mechanism proposed. We are led, then, to two conclusions:

1. L-glutamate forms a ternary complex which hinders TPNH leaving, thereby suppressing the dissociation of the enzyme–TPNH complex.
2. L-glutamate does not change the fluorescence of the enzyme–TPNH complex itself, but only causes an increase in the fluorescence intensity of the solution by increasing the number of molecules of enzyme-bound TPNH in that solution.

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Sur la structure chimique de la partie peptidique de la cire D d'une souche humaine de *Mycobacterium tuberculosis*

Les souches humaines de *M. tuberculosis* contiennent un peptido-glycolipide appelé cire D, qui peut remplacer les Mycobactéries dans l'adjuvant de FREUND[1]. L'activité

Abbréviations: DAP, acide diaminopimélique; Glu, acide glutaminique; Ala; alanine; DNP, dinitrophényl-.

adjuvante semble liée à la présence d'une partie peptidique, car les cires D de souches bovines de *M. tuberculosis* qui ne contiennent pas d'acides aminés sont inactives comme adjuvants. La cire D de la souche humaine virulente "Brévannes" contient 7 molécules d'acides aminés (pour un poids moléculaire de 54,000): 2 *meso*-DAP, 2 D-Glu, 2 L-Ala et 1 D-Ala[2]. La présence d'acides aminés de configuration D est remarquable et semble assez générale pour des peptido-lipides et peptido-glycolipides des Actinomycetales[3]. La présente note étudie la structure chimique de la partie peptidique et les liaisons existant entre les parties peptidique et glucidique.

La partie hydrosoluble de la cire D a d'abord été dessalifiée sur Amberlite MB 3. Une chromatographie sur résine Dowex 50 × 2 a montré que le produit semble homogène.

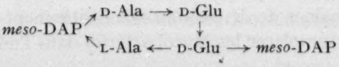
Structure chimique de la partie peptidique

Comme la plupart des acides aminés sont présents sous la forme D, il n'était pas possible de faire appel à des enzymes protéolytiques pour établir la structure chimique. Des essais d'hydrolyse par la pepsine se sont d'ailleurs révélés infructueux. La cire D a été soumise à des hydrolyses acides partielles (HCl 6 *N*; 37°) de durées variables (20, 60, 72, 172 h) et les produits de réaction ont été séparés par chromatographie sur colonne de Dowex 50 × 2. Lorsque la durée de l'hydrolyse dépasse 60 h, on caractérise toujours 5 pics principaux.

Le pic 1 ne contient que des sucres, le pic 4 que des traces de produit. Les différentes fractions constituant un pic ont été réunies puis dessalifiées soit sur Dowex 2 × 8 (pics 2, 3, 3bis) soit sur Dowex 50 × 2 (pic 5)[4]. La pureté des pics a été vérifiée par chromatographie sur papier Whatman No. 1 et lorsque cela s'est révélé nécessaire, des chromatographies préparatives sur papier ont achevé la purification. Le Tableau I donne une liste des différents peptides obtenus. Les R_F ont été établis dans le solvant: *n*-butanol–acide formique–eau (75:15:10, v/v/v); les acides aminés N-terminaux ont été déterminés par la méthode de SANGER[5] et la configuration optique de l'alanine par la méthode de WOOD ET GUNSALUS[6] modifiée par SZULMAJSTER[7].

Le Tableau I montre aussi comment, grâce aux peptides obtenus par hydrolyse acide partielle, on peut envisager d'attribuer à la partie peptidique de la cire D la structure d'un heptapeptide linéaire (I). Mais comme par la méthode de Sanger on n'a pas pu obtenir de *meso*-di-DNP-DAP sur la cire D initiale, et comme de plus

TABLEAU I

Pic	*Peptide*	R_F	*Structure des peptides obtenus par hydrolyse partielle*
2	a	0	D-Glu.(L-Ala,*meso*-DAP) + sucres
2	b	0.03	D-Glu.(L-Ala,*meso*-DAP,L-Ala)
3	a	0.31	D-Glu.Ala
2	c	0.16	D-Glu.D-Glu
3bis	a	0.31	D-Ala.D-Glu
5	c	0.08	*meso*-DAP.D-Ala
		(I)	*meso*-DAP.D-Ala.D-Glu.D-Glu.L-Ala.*meso*-DAP.L-Ala
		(II)	 *meso*-DAP D-Ala D-Glu L-Ala D-Glu *meso*-DAP

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* Contrairement à ce que nous avions observé précédemment[2].

la carboxypeptidase n'a pas pu détacher la L-alanine en position terminale*, il semble que les deux groupements aminés du *meso*-DAP N-terminal et le groupement carboxylique de l'alanine C-terminale soient impliqués dans des liaisons non encore déterminées. On peut alors envisager une structure cyclique (II) qui tient également compte de tous les peptides partiels isolés. Dans le cyclopeptide (II) l'un des résidus de *meso*-DAP porte sur l'un de ses carboxyles la deuxième molécule de L-Ala.

Liaisons entre les parties peptidique et glucidique

Lorsque l'on dessalifie les fractions correspondant au pic 2 non plus sur Dowex 2 × 8 mais sur Dowex 50 × 2, on peut caractériser 2 substances A et B ayant respectivement dans le *n*-butanol acide des R_F de 0 et 0.03 et qui, après hydrolyse acide totale (HCl 6 *N*; 18 h, 110°) libèrent du *meso*-DAP (R_F dans le même solvant du DAP, 0.05). Après une hydrolyse plus douce (HCl 1 *N* ou 2 *N*; 2 ou 4 h; 110°) il a été possible de déceler la présence de galactosamine et de D-arabinose dans A, de galactosamine dans B. Si A ou B sont d'abord soumis à l'action du dinitrofluorobenzène puis à une hydrolyse acide il est possible de caractériser du di-DNP-*meso*-DAP et de la galactosamine. Une des liaisons entre les parties peptidique et glucidique de la cire D est donc constituée par une *liaison entre un groupement carboxylique du meso-DAP et la galactosamine*. Cetter dernière à son tour est liée au D-arabinose. Il n'a pas encore été possible de préciser la nature de la liaison DAP-galactosamine ni duquel des 2 résidus de *meso*-DAP il s'agit.

Une *deuxième liaison* entre les parties peptidique et glucidique a pu être caractérisée grâce à l'étude d'un hydrolysat acide partiel de cire D de 20 h seulement. Après chromatographie sur Dowex 50 × 2 on obtient deux pics. Dans le premier il a été possible d'isoler un glycopeptide (R_F, 0). La structure de la partie peptidique est: Ala.Glu, et de plus on a caractérisé du D-arabinose et du D-galactose. Nous sommes donc en présence d'une liaison ester entre le groupement carboxylique de l'acide glutamique (résidu No. 3 de l'heptapeptide) et un groupement hydroxyle, soit de l'arabinose soit du galactose.

A l'heure actuelle il n'est cependant pas encore possible d'affirmer si les deux liaisons entre les parties peptidique et glucidique qui viennent d'être signalées sont les seules ou s'il en existe d'autres.

Nous remercions vivement le Dr. SZULMAJSTER d'avoir bien voulu nous aider pour la détermination des résidus L- et D-alanine.

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A new enzymic reaction concerned in the metabolism of γ-hydroxyglutamic acid

γ-Hydroxyglutamic acid is known as a metabolic product of L-hydroxyproline in rat liver[1]. Recently DEKKER[2] has reported the enzymic formation of glyoxylic acid from γ-hydroxyglutamic acid. In the present study we report the isolation from rat liver of a condensing enzyme which forms γ-hydroxy-α-ketoglutaric acid from pyruvic acid and glyoxylic acid. The enzyme was purified about 12-fold from an extract of an acetone powder of rat liver by fractionation with ammonium sulfate and acetone The acid was separated from other keto acids as 2,4-dinitrophenylhydrazone by paper chromatography. The stoichiometry (Table I) shows that one mole of the keto acid is produced from one mole of pyruvic acid and one mole of glyoxylic acid. The keto acid was isolated from the reaction mixture as the calcium salt and the product of reductive amination of the acid with Raney-Ni + H_2 + NH_3 was shown to be γ-hydroxyglutamic acid by paper chromatography with four different solvent systems. γ-Hydroxyglutamic acid was also formed from the 2,4-dinitrophenylhydrazone of the acid by catalytic reduction with Pt + H_2 (ref. 3). Moreover, the keto acid was decarboxylated with ceric sulfate, which is generally used for α-keto acid decarboxylation, and the existence of the hydroxyl group of the acid was proved by the xanthate-molybdate test and the sulfur test[4] for secondary alcohols. From these results it may be concluded that the product of the enzymic reaction is γ-hydroxy-α-ketoglutarate. When the calcium-salt of the keto acid was used as substrate of the condensing enzyme, it was split into pyruvic acid and glyoxylic acid in equimolar amounts, which was the evidence that the reaction is reversible. Formaldehyde or acetaldehyde did not react with pyruvic acid or glyoxylic acid in the presence of the enzyme, which distinguishes the latter from the formaldehyde and pyruvic acid condensing enzyme reported by HIFT AND MAHLER[5]. γ-Hydroxy-α-ketoglutaric acid was formed also from authentic γ-hydroxyglutamic acid by the action of a transaminase obtained from the same source by ammonium sulfate fractionation (62–75 %). The transaminase preparation was completely free of the condensing

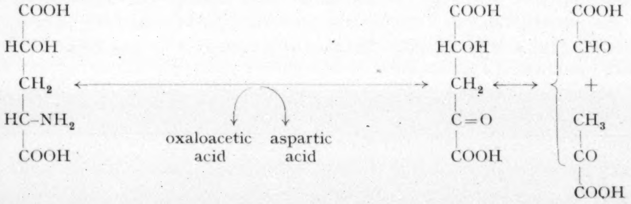
TABLE I

THE BALANCE SHEET OF THE ENZYMIC CONDENSING REACTION AND ITS REVERSIBILITY

The reaction mixture (3.0 ml) contained 30 μmoles potassium phosphate buffer (pH 7.4), 5 μmoles sodium glyoxylate, 5 μmoles lithium pyruvate, or 5 μmoles γ-hydroxy-α-ketoglutaric acid (obtained enzymically as calcium salt and standardized from its crystalline 2,4-dinitrophenylhydrazone), enzyme protein, 1 mg. Gas phase, N_2. Incubated for 60 min at 37°. There were little changes in the amounts of enzymic products, when air was used for gas phase during incubation. The amount of the product was measured as its 2,4-dinitrophenylhydrazone, after being extracted from the reaction mixture by ethyl acetate and separated by paper chromatography.

	The condensing reaction of pyruvic acid and glyoxylic acid			*The splitting reaction of γ-hydroxy-α-ketoglutaric acid*		
	Before incubaiton (μmoles)	*After incubation (μmoles)*	*Difference (μmoles)*	*Before incubation (μmoles)*	*After incubation (μmoles)*	*Difference (μmoles)*
Glyoxylic acid	5.0	2.70	2.30	—	2.47	2.47
Pyruvic acid	5.0	2.81	2.19	—	2.55	2.55
γ-Hydroxy-αKeto-glutaric acid	—	2.22	2.22	5.0	2.39	2.61

enzyme. In the transaminase reaction, the acceptors of the amino group of γ-hydroxyglutamic acid were oxaloacetic acid or α-ketoglutaric acid, from which aspartic acid or glutamic acid was formed respectively. Formation of amino acids was confirmed by paper chromatography with four different solvent systems. γ-Hydroxy-α-ketoglutaric acid formed by the transaminase had the same properties as the keto acid formed by condensation of pyruvic acid and glyoxylic acid enzymically, and was split into pyruvic and glyoxylic acid in equimolar amounts by adding the condensing enzyme (free of the transaminase). Accordingly, one of the metabolic pathways of γ-hydroxyglutamic acid in rat liver may be shown as follows:



Authentic γ-hydroxyglutamic acid was generously donated by Prof. S. AKABORI and Prof. T. KANEKO, Faculty of Science, Osaka University, Osaka. This work was supported in part by grants for scientific research from the Ministry of Education.

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Calcification *in vivo* of implanted collagen

On the basis of nucleation experiments[1-3] *in vitro*, it has been suggested that formation of the mineral phase of bone is initiated by an interaction between calcium, phosphate and some as yet unidentified template on the collagen polymer. It has further been postulated that inhibitors[1] or promoters[5] of calcification, or differences in the reactivity of hard- and soft-tissue collagens[6], may be factors determining which of the collagenous tissues do or do not undergo mineralization. None of these ideas have been substantiated by evidence gained from experimentation *in vivo*.

In recent studies carried on as part of an investigation of collagen catabolism, we observed that rat-tail tendon and reconstituted collagen implanted in the peritoneal cavities of rats became calcified. The possibility of using a peritoneal implant technique for studies *in vivo* of the mechanism of calcification was recognized, and the exploratory experiments described here were undertaken. Collagen preparations of several types were implanted into rats and rabbits, and the material was recovered at various intervals for chemical analysis, and for electron microscopy and diffraction. The specimens destined for electron microscopy and diffraction were fixed in an OsO_4–$K_2Cr_2O_7$ mixture, embedded in methacrylate, and sectioned on a Porter-Blum microtome with glass and diamond knives.

Strips of tail tendon from mature rats were implanted into the peritoneal cavities of female Sprague-Dawley rats weighing 100–150 g. Samples of tendon removed up to 30 days appeared unchanged under the electron microscope, did not give electron-diffraction patterns and had essentially the same calcium: hydroxyproline ratio as the material before implantation. Samples removed at 100, 120 and 160 days were

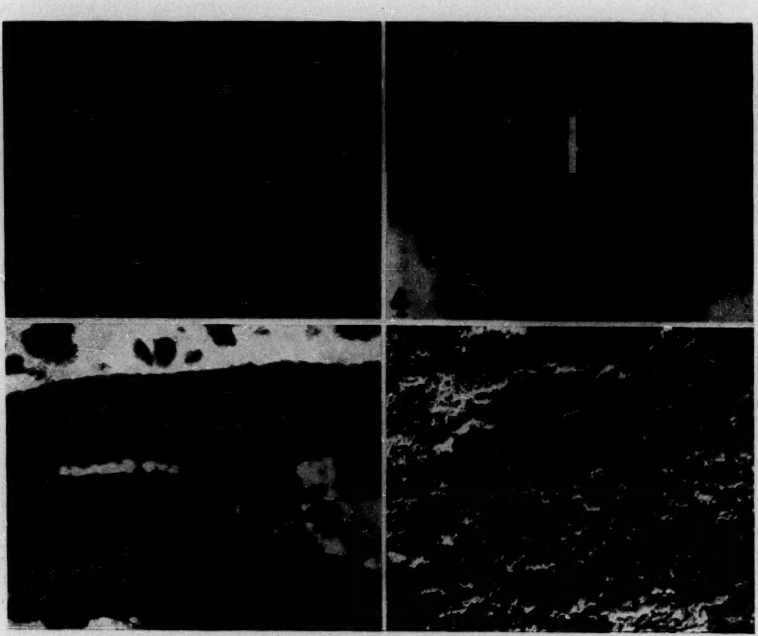

Fig. 1. The edge of a calcified region in a 100-day tendon implant, showing crystals aligned in correspondence to the collagen striation pattern. 50,000 ×.

Fig. 2. An 18-day direct implant of reconstituted collagen, in which the striation pattern is almost entirely masked by mineral. 40,000 ×.

Fig. 3. A standard transmission diffraction pattern obtained from a relatively large area of cross-sectioned fibrils from the same direct implant of reconstituted collagen. Unoriented patterns made in this fashion demonstrate clearly that the mineral present is apatite.

Fig. 4. A selected area diffraction pattern obtained from longitudinally sectioned fibrils in a different region of the preceding preparation. Preferred orientation of the crystals is evident.

Fig. 5. A very heavily mineralized area of reconstituted collagen implanted 160 days in a dialysis bag. Individual fibrils and crystals can no longer be distinguished. 35,000 ×.

found to be hard on gross examination, and marked calcification was indicated by the typically altered calcium: hydroxyproline ratio, and by the presence in the collagen fibrils of microscopically visible crystals, which were identified by diffraction as hydroxyapatite. The crystals were obviously laid down in an array conforming to the pattern established by the periodic spacings of the fibrils (Fig. 1), and the diffraction patterns further indicated that they were preferentially oriented, with their c-axes paralleling the fibril axes. Tail tendon dissected from these same animals at the times when calcified tendon implants were removed showed no evidence of mineralization.

Reconstituted rabbit-skin collagen, in the form of heat-precipitated gels, was implanted either directly or in dialysis bags into the peritoneal cavities of 2-kg female New Zealand white rabbits and 100–150-g female Sprague-Dawley rats. The collagen gels were precipitated at pH 7.4 in either 0.16 *M* NaCl or 0.4 I phosphate buffer. The fibrils reconstituted in these two solutions appeared identical under the electron microscope, and showed the 640-Å periodicity characteristic of native collagen. Mineralization of the directly implanted gels prepared with the phosphate buffer was observed after as short a period as 16 days in both the homologous (rabbit) and heterologous (rat) animals (Fig. 2). Crystals were again seen on the surfaces of and within the fibrils of all the reconstituted collagen implants which calcified. The diffraction patterns indicated that these crystals were also hydroxyapatite (Fig. 3), and that they were likewise oriented with their c-axes paralleling the fibril axes (Fig. 4). Throughout the 160-day course of the experiment, calcification was not detected in the directly implanted gels of collagen reconstituted from the NaCl solutions. Although the results were erratic, some of the gels prepared from NaCl solutions and implanted in dialysis bags were found to be calcified at the end of this period (Fig. 5).

These preliminary results indicate that normally non-mineralizing collagen fibrils can be induced to calcify in an ordered pattern through transplantation to the peritoneal cavity either in their natural state or after purification by means of reconstitution techniques. Challenging possibilities now arise for experimentation *in vivo* with collagen preparations which may be subjected to various treatments prior to implantation, or implanted in combination with agents thought to influence mineralization.

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Enzymic conversion of *p*-tyramine to *p*-hydroxyphenylethanolamine (norsynephrin)

With the discovery of norsynephrin and synephrin (N-methylnorsynephrin) in urine[1-3] and of the former in animal tissues[1], it became of interest to study their biosynthesis. It has now been possible to demonstrate that the enzyme 3,4-dihydroxyphenylethylamine (dopamine) β-oxidase is not specific for dopamine but can catalyze the oxidation of *p*-tyramine to norsynephrin. In addition, the simplicity and specificity of the method for the determination of norsynephrin, in the presence of its precursor, make possible a simplified procedure for measuring dopamine β-oxidase activity.

In a typical experiment 0.5 g of slices were suspended in 3.0 ml Krebs–Ringer bicarbonate buffer, pH 7.4, and preincubated at 37° with 300 μg of the monoamine oxidase inhibitor, harmaline, for 5 min. 30 μmoles *p*-tyramine were then added and the incubation was continued for 30 min in an atmosphere of 95 % O_2–5 % CO_2. The reaction was stopped by adding 3.0 ml 12 % trichloroacetic acid and the slices were homogenized in the mixture before centrifugation.

Assay of norsynephrin. Periodate quantitatively oxidizes norsynephrin but not tyramine to *p*-hydroxybenzaldehyde. The latter compound, when dissolved in NH_4OH, has a sharp absorption peak at 330–333 mμ which serves as the basis for the determination of the enzymically formed norsynephrin. The deproteinized incubation mixture is passed through a 0.6 × 3 cm Dowex-50 H^+ column. After washing the column with 5–10 ml water, the adsorbed bases are eluted with 3 ml 3 *N* NH_4OH and the eluate is collected in a 40-ml extraction tube. Norsynephrin is then converted to *p*-hydroxybenzaldehyde by the addition of 0.3 ml 2 % $NaIO_4$. After 3 min, excess periodate is reduced with 0.3 ml 10 % $NaHSO_3$. The benzaldehyde is extracted from the solution with 30 ml toluene, and an aliquot of the solvent is re-extracted with 1 ml 3 *N* NH_4OH. Absorbancy is measured at 330 mμ and compared with a norsynephrin standard* carried through the entire procedure.

As shown in Table I β-oxidation of tyramine was found to be most active in adrenal medulla, less activity being present in brain and little in liver. To show that the enzymically formed material was norsynephrin, several incubation flasks with adrenal-medulla slices were pooled and aliquots were taken for various analyses.

TABLE I

HYDROXYLATION OF TYRAMINE BY TISSUE SLICES

Tissue	*Norsynephrine formed* (*μg/g/h*)
Beef adrenal medulla	39.7
Hog-brain hypothalamus	4.6
Hog-caudate nucleus	3.9
Hog cortex and cerebellum	1.7
Whole rat brain	1.3
Rat liver	0.7

Slices (0.5 g) were suspended in 3.0 ml Krebs–Ringer bicarbonate buffer, pH 7.4, and preincubated at 37° with 300 μg harmaline for 5 min. 30 μmoles *p*-tyramine were then added and the incubation was continued for 30 min in an atmosphere of 95 % O_2–5 % CO_2.

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* Norsynephrin was generously supplied by Dr. SIDNEY ARCHER of Sterling Winthrop Research Institute.

The product obtained on treatment with periodate was found to have the identical spectrum of *p*-hydroxybenzaldehyde. Chromatography of the experimental incubation mixtures in *n*-butanol–acetic acid–water (50:12:50, butanol layer) and in ethanol–conc. NH_4OH–water (180:10:10) revealed a new spot having the same R_F values as authentic norsynephrin and yielding the same colors with spray reagents; pink with diazotized *p*-nitroaniline, yellow with diazotized sulfanilic acid, and ultraviolet adsorption with 1 % periodate in 5 % Na_2CO_3 (Fig. 1). In another experiment with similar amounts of adrenal-medulla slices and [^{14}C]tyramine, the norsynephrin was separated from the tyramine by one-dimensional chromatography in the butanol solvent and rechromatographed in two dimensions using the solvents isopropanol–conc. NH_4OH–water (200:10:20) and *n*-butanol–acetic acid–water (120:30:50).

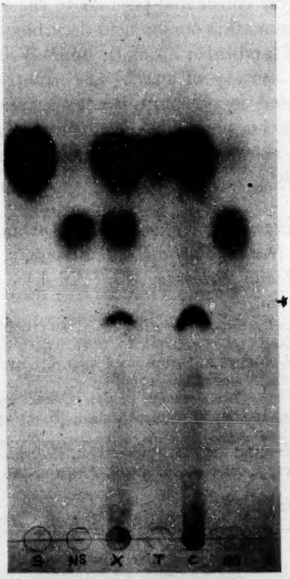

Fig. 1. Paper chromatographic evidence for norsynephrin formation with adrenal medulla slices. "S" signifies authentic synephrin; NS, norsynephrin; X, incubation mixture; T, tyramine; C, zero-time incubation. The solvent used was ethanol–conc. NH_4OH–water (180:10:10) and the spray reagent was diazotized *p*-nitroaniline.

The radioactivity and norsynephrin color reactions coincided in one spot, which migrated exactly as authentic norsynephrin. Furthermore, when the enzymically formed norsynephrin was treated with periodate, it yielded one detectable radioactive product which could be extracted with toluene. This was characterized* as *p*-hydroxybenzaldehyde by the use of gas chromatography, using a 6-ft. column composed of 12 % ethylene glycol–adipate polyester on Chromosorb-W coupled to a scintillation counter[4].

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* We wish to thank Dr. Arthur Karmen for these measurements.

The following findings indicate that the enzyme responsible for norsynephrin formation is dopamine β-oxidase. First, adrenal-medulla particles prepared according to LEVIN *et al.*[5] were found to have similar requirements for norsynephrin formation as for norepinephrine formation. Ascorbate, fumarate and adenosine triphosphate all stimulated and KCN inhibited the hydroxylation. Secondly, dopamine was found to inhibit the oxidation of tyramine in a competitive manner. Finally, the localization of activity in adrenal medulla and brain-stem areas were also characteristic of dopamine β-oxidase[6].

Norsynephrin was first isolated from octopus salivary glands by ERSPAMER[7] who called it octopamine. Until its more recent finding in human and animal urine and in tissues, it was merely considered peculiar to the octopus. However, it is now apparent that a general aromatic L-amino acid decarboxylase in mammalian tissues[8] makes available tyramine (and other aromatic amines) for β-oxidation. The activity of the β-oxidase with a variety of amines is currently under investigation. The significance of synephrin and norsynephrin is not apparent, but it should be pointed out that they are fairly active pharmacologic agents[9]. Furthermore, as pointed out by ERSPAMER[7], they are readily oxidized by chemical means to epinephrine and norepinephrine respectively.

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Biological activities of isomeric estriols

In a comprehensive study of the estrus-producing values of a large number of steroidal estrogens, we have employed in the St. Louis University laboratories both the Mather Modification[1] of the MARRIAN–PARKES[2] assay procedure using the adult castrate mouse and the CURTIS–DOISY[3] method using the 20-day-old intact female rat, as we believe that these two assay methods are suitable for the comparative study of estrogens of widely varying potencies. An average of about 50 animals was used for each dose level of each estrogen in obtaining dose-response curves.

As shown in Table I, the three isomeric estriols were surprisingly estrogenic. Especially noteworthy is the fact that 17-epiestriol in the CURTIS–DOISY assay is

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substantially more active than estradiol-17β. PRELOG, RUZICKA AND WIELAND[4] reported the estrus-producing potency of 17-epiestriol as only 5–10 μg per rat unit in the adult castrate rat using the ALLEN–DOISY assay, and this isomeric estriol has never been considered as a very potent estrogen[5]. This is the first time, to our knowledge, that a naturally-occurring estrogen has been shown to be more estrogenic than estradiol-17β. This finding could conceivably account for the unexplainably high estrogenic titers found in human pregnancy urine at times[6]. BREUER[7] has reported that 17-epiestriol results from the incubation of 16α-hydroxyestrone with human-liver slices, from the incubation of 16-keto-estrone with human liver, ovary, and kidney slices, and more recently he has described its isolation from human pregnancy urine[8].

TABLE I

Estrogen	MARRIAN *Assay (μg giving 50 % response)*	CURTIS–DOISY *Assay (μg giving 50 % response)*	*% Variation from normal in 21-day-old male rats* (50 μg/day for 14 days)*		
			Body wt. gain	*Ventral prostate (wt.)*	*Testes (wt.)*
Estradiol-17β	0.02	0.09	— 38	— 53	— 50
Estriol	0.1	0.13	— 39	— 63	— 45
16-Epiestriol	0.2	0.2	— 32	— 72	— 37
17-Epiestriol	0.044	0.054	— 42	— 72	— 55

* Cf. ref. 10.

In Table I are also shown certain data extracted from our general screening assays employing young intact male rats, as performed by the Endocrine Laboratories of Madison, Wisc. under the direction of Dr. ELVA G. SHIPLEY. It is apparent that all of the estriols exert a very strong dampening effect on the growth of the ventral prostate. It appears that 16-epiestriol, which has been termed one of the weakest of all naturally-occurring estrogens[9], may produce in the young male rat a disproportionate suppression of pituitary gonadotrophin relative to estrus-producing potency.

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